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edited by

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C. FRED FOX

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DNA REPAIR MECHANISMS

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Preface

This volume documents the proceedings of a major international conference on DNA Repair Mechanisms, held at Keystone, Colorado in February 1978. The meeting was one of the 1978 ICN–UCLA Winter Symposia on Molecular and Cellular Biology, sponsored by ICN Pharmaceuticals, Inc., and organized through the Molecular Biology Institute of the University of California, Los Angeles.

The conference marked the fourth anniversary of the first comprehensive international meeting on the subject of DNA repair held at Squaw Valley, California in 1974. That meeting generated a great deal of enthusiasm and catalyzed many significant collaborative ventures among the nearly 200 participants. In the ensuing years the field has more than doubled in number of researchers and in the rate of proliferation of new information and publications on the subject. This surge of interest is due in part to the increased evidence that many types of repairable damage in DNA are also highly mutagenic and carcinogenic. Procedures for measuring the repair of damaged DNA have become refined and new ones have been developed in the past several years. In addition, new pathways for the repair of DNA damage by excision have been discovered and error-prone bypass modes have been elucidated. Enough new repair enzymes have been isolated and characterized that it has become imperative that a rational nomenclature be developed. Much more is known about the hereditary disease *Xeroderma pigmentosum* with respect to its deficiency in several DNA repair modes and evidence for repair defects in other human hereditary diseases is being established. DNA repair currently represents one of the most active fields of investigation in the overlapping spheres of molecular biology and clinical research on human genetic deficiency and aging.

The 1978 meeting was attended by nearly 400 participants. The format for the meeting included some novel aspects as well as the more traditional symposium style. An overall perspective on the field of DNA repair was provided in plenary sessions in which 17 speakers outlined current areas of active investigation and important problems in the field. Then, selected questions formed the focus of 13

workshop sessions coordinated by designated experts representing various views and approaches to the respective problems. Poster sessions were utilized to facilitate the presentation of new data from additional participants.

It is important that researchers in DNA repair periodically have the opportunity to gather at a conference of this sort. The field includes scientists in the diverse disciplines of biochemistry, genetics, photobiology, radiology, and environmental biology. Workers in these disciplines are affiliated with different national and international scientific societies and the field of DNA repair derives benefits from these multiple associations. However, it also requires multidisciplinary gatherings such as this conference to promote the free interchange of information essential to the advancement of the field.

This volume includes papers from the plenary session speakers and the workshop participants as well as short summaries by the workshop session conveners, describing the current state of agreement and dispute on specific topic areas. We have additionally included a number of manuscripts from a selected group of poster contributors. Difficult decisions had to be made because of the space limitations in the volume and we sincerely regret that we were unable to include papers from more of the excellent poster presentations. In choosing these we tried to include documentation of important new findings, theories, and approaches to complement the material covered in the other papers and have, wherever possible, stressed the availability of multiple biological systems of DNA repair and mutagenesis research. Finally, the volume includes contributions from the two keynote lecturers, Paul Howard-Flanders and Bruce Ames.

We have arranged the papers in the volume roughly according to their organization in the meeting, with an attempt to group plenary session contributions together with the relevant workshop and poster manuscripts. We hope that the result represents a logical and comprehensive treatment of the rapidly moving field of DNA repair as of early 1978.

We wish to acknowledge the financial sponsorship for this meeting provided predominantly by contracts with the U.S. Department of Energy and by the National Institutes of Health (in particular the National Cancer Institute, National Institute on Aging, National Institute on Child Health and Development, National Institute on Allergies and Infectious Diseases and the Fogerty International Center). We also solicited support from many major chemical and drug companies because of the obvious relationship of this conference topic to carcinogenic chemicals in the environment. We are pleased that the following companies were able to respond with support and we appreciate the commitment to scientific enquiry and concern expressed by these private organizations: E. I. du Pont de Nemours and Co., Merck and Co., Eli Lilly, and Hoffman–La Roche.

We appreciate the excellent suggestions and help we received from many of our colleagues in assembling the program for this meeting. Finally we are indebted to Fran Stusser and her staff and to the Keystone administration for their generally efficient handling of the arrangements for the meeting.

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REPAIRABLE DAMAGE IN DNA[1]

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ABSTRACT

Important contributions to the elucidation of the role of DNA repair for cell viability, mutagenesis, malignant transformation and cell degeneration are expected from experiments in which individual DNA lesions of known chemical structure are investigated. This is important in view of the high degree of complexity of the spectrum of lesions which is induced by most physical and chemical agents by *direct action* and by *indirect action* via the intermediacy of active oxygen species. The formation of thymine damage in baby hamster kidney cells by indirect action was demonstrated for the chemical DNA damaging agents benzo(a)pyrene (B(a)P) and ascorbic acid/Cu^{2}+. It is useful to characterize the biochemical properties of DNA lesions in terms of their *excisability, coding properties* and *regulatory properties*. As a start the excisability of several structurally related arylation products of guanine induced by B(a)P and N-acetoxy-acetylaminofluorene (N-acetoxy-AAF) was determined in the epitheloid human alveolar tumor line A549. The following decreasing scale of excisability was observed: B(a)P-4,5-epoxide adduct C_2 > B(a)P-4,5-epoxide adduct C_1 >> B(a)P-diol-epoxide II-deoxyguanosine > deacetylation product of "major" deoxyguanosine-AAF adduct > B(a)P-diol-epoxide I-deoxyguanosine > "major" deoxyguanosine-AAF adduct ≥ "minor" deoxyguanosine-AAF adduct. For the B(a)P-deoxyguanosine adducts these results suggest an inverse relationship between the excisability of the lesions and the mutagenicity and toxicity of the B(a)P-metabolites by which they are induced in mammalian cells. The effect of the location of the lesions in chromatin on their excisability is discussed.

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INTRODUCTION

The elucidation of the role of the repair of DNA damage in cell viability, mutagenesis, malignant transformation and cell degeneration represents a major goal in molecular toxicology. Important contributions to this goal are expected from experimental systems in which *individual DNA lesions* of known chemical structure can be investigated. The study of *DNA damaging agents* which induce a whole spectrum of lesions and the use of biochemical procedures which do not allow the distinction of individual lesions are less informative. The biochemical and biological effects resulting from the treatment of a cell with a DNA damaging agent are usually a composite of the expression of a multitude of lesions and it is often very difficult or impossible to dissect the contributions made by individual lesions. However, it is the chemical structure of the individual lesions, their abundance and distribution in the genome which determine the mode(s) of lesion processing and ultimately the biological endpoints in a particular cell. *Our "short" range goal should be to define the toxicity, mutagenicity and transforming potency of individual lesions rather than of damaging agents.* With the help of this knowledge it may become possible to analyze multiple lesion situations where lesion interaction may influence repairability and biological expression.

In this article I will first discuss lesion formation and point out the complexity of the spectrum of lesions which are formed in the DNA of intact cells by most physical and chemical damaging agents. I will then describe and define biochemical properties of DNA lesions and, as an example, I will review our present knowledge of the relative excisability of a series of structurally related arylation lesions of guanine. In a final paragraph I will discuss the effects which the intragenomic location of lesions may have on their repairability.

(1) FORMATION OF DNA LESIONS BY DIRECT AND INDIRECT ACTION

The list of DNA damaging agents is long and the list of DNA lesions which they produce is even longer. There is hardly an atom on the purine and pyrimidine rings of DNA which has not been shown to react with some physical or chemical damaging agent. Often it is possible to predict the type of lesions which are likely to be formed from basic chemical principles, e.g. a majority of ultimate chemical damaging agents possess electrophilic properties and react with the electron-rich centers of the heterocyclic bases in nucleophilic displacement reactions[1]. Valuable information

about the structure of DNA lesions can be obtained from *in vitro* experiments with mono- and polynucleotides but reliable data concerning the relative abundance and distribution of the lesions *in situ* can only be obtained in experiments with intact cells. Most experiments with chemical agents use highly radioactive compounds in order to be able to detect the introduction of radioactive substituents on the DNA bases (or the backbone) at low levels of damage. However, an important class of DNA lesions escapes detection under these conditions. There are a number of chemical DNA damaging agents of considerable importance to biochemical and medical research which may exert at least part of their action via the formation of reactive oxygen species. Most of these agents, e.g. 4-nitroquinoline-N-oxide[2], streptonigrin[3], adriamycin, mitomycin C[4], benzo(a)pyrene (B(a)P)[5], possess or are metabolically activated to quinone-like structures. In many cell systems they can participate in one-electron redox cycles which lead to the formation of highly reactive oxygen radical species such as superoxide- and hydroxyl-radicals which are expected to induce DNA lesions of the type produced by radiation. Oxygen radical species may also be responsible for the chromosomal damage induced by ascorbic acid plus Cu^{2+}. The formation of DNA strand-breakage by some of these agents has been clearly demonstrated in *in vitro* component systems[7] but not in intact cells where drug-induced breaks have to be distinguished from breaks introduced as a consequence of DNA repair.

Hydroxyl- and superoxide- radicals are formed as a consequence of water radiolysis by ionizing radiation. Hydroxyl radicals have been shown to induce thymine damage[8,9] and DNA strand breaks[10] in intact cells and they are mostly responsible for the lethal action of aerobic gamma- and x-rays[11]. This type of radiation action which is mediated via oxygen radical species is referred to as "indirect action". In contrast, energy deposition into the DNA target is referred to as "direct action" of ionizing radiation. Similarly, active oxygen species are also formed by far-ultraviolet light by water photolysis and by near-ultraviolet light by photodynamic action and photodissociation of hydrogen peroxide[12,13]. The type of DNA lesions formed by active oxygen species is expected to be closely related regardless of whether they are formed by ionizing radiation, ultraviolet light or in a redox system involving a chemical agent. Therefore, it may be useful to expand the terminology developed for ionizing radiation to include ultraviolet light and chemical agents and we are proposing the following definitions:

DIRECT ACTION: attack of DNA by the primary agent or a chemical derivative of the primary agent.

INDIRECT ACTION: (of ionizing radiation, ultraviolet radiation or a chemical) attack of DNA by active oxygen species which are formed by the reaction of the primary agent with a non-DNA target.

In general, direct action is expected to predominate for most chemical damaging agents and for far-ultraviolet light while indirect action represents the major intracellular reaction mechanism for ionizing radiation. There is great variation in the structure of DNA lesions induced by direct chemical action. In contrast, a similar spectrum of lesions is expected to be introduced by indirect action of physical and chemical agents. They include: single- and double-strand breaks, products of the 5,6-dihydroxy-dihydrothymine type, damage at the thymine - methyl group, damage involving the other DNA bases, DNA-DNA, DNA-RNA and DNA-protein cross links.

Evidence for indirect action of ultraviolet light and the chemical damaging agents ascorbic acid/Cu^{2+} and benzo-(a)pyrene (B(a)P) in intact mammalian cells has recently been obtained in our laboratory. The formation of products of the 5,6-dihydroxy-dihydrothymine type (t^{uv}) and the abstraction of tritium from thymine-methyl [^{3}H] was observed in HeLa cells upon irradiation with monochromatic light at 240, 260, 280, 313 and 365 nm. The efficiency of formation of t^{uv} type products was comparable to thymine photodimerization in the near ultraviolet at 313nm[12]. There is little doubt that these lesions are formed by indirect action of ultraviolet light. The formation of [^{3}H]H_2O in baby hamster kidney cells (BHK) which had been prelabeled in their DNA with thymine-methyl[^{3}H] was observed upon treatment with $2x10^{-3}$M ascorbic acid/$2x10^{-5}$M Cu^{2+}. It was estimated that after 4 hours of incubation approximately 0.2% of all thymine-methyl groups had reacted with hydroxyl-radicals yielding 5-Methylene-uracil radicals and water (M. Ide and P. Cerutti, unpublished). 5-methylene-uracil radicals may in part be repaired chemically to thymine by hydrogen abstraction from neighboring organic molecules and participate in secondary radical chain reactions which may result in DNA strand breakage and DNA cross-linking. BHK cells have retained the capacity in culture to metabolize B(a)P. Polycyclic aromatic hydrocarbons are in part metabolized by one-electron oxidation reactions. The 6-oxo-B(a)P-radical represents an important intermediate which is further oxidized to the stable metabolites 6,12-, 1, 6- and 3,6-B(a)p-dione. Lorentzen and Ts'o[5] have speculated that the

B(a)P-diones might participate in redox cycles and that superoxide radicals, hydrogen peroxide and hydroxyl-radicals might be formed as a consequence. Preliminary results of experiments similar to those described above for the ascorbic acid/Cu^{2+} system support this hypothesis and suggest the formation of DNA-base damage by indirect action of B(a)P[14].

In summary, the spectrum of lesions induced by most DNA damaging agents is extremely complex. *Both physical and chemical agents are capable of producing lesions by direct and indirect action.* Lesions induced by indirect chemical action may escape detection when standard experimental protocols are followed. *In studies of DNA repair and its relationship to the biological effects of DNA damage it is of greatest importance that agents are chosen which induce a simple spectrum of structurally characterized lesions.* The biochemical characterization of individual lesions is discussed in the following section.

(2) BIOCHEMICAL PROPERTIES OF DNA LESIONS

The biological expression of a lesion is mediated by its biochemical properties which in turn are determined by its chemical properties. The chemical structure of individual lesions is, of course, independent of the cell system in which they are being studied but their biochemical properties and biological expression are not. Therefore, questions concerning lesion chemistry can often be adequately studied *in vitro* but the biochemical and biological properties of lesions have to be investigated in the intact cell. Major chemical properties of lesions are their electronic configuration, hydrogen bonding potential, stereochemistry and solvation and their effect on local DNA- and chromatin-conformation. Major biochemical properties include excisability, persistence, coding properties and regulatory properties. It may be useful to describe these terms for our present discussion.

EXCISABILITY: Efficiency by which a lesion is removed from the genome in a particular cell. Excisability is expressed as the fraction (and amount in μmoles lesion per mole DNA-phosphate) of a specific lesion removed from the genome in one generation under non-saturating conditions. Under *non-saturating conditions* the fraction of the lesions removed per time unit is independent of the initial lesion concentration. Several pathways, such as nucleotide-excision and base-excision, contribute to total excisability and it may be possible to assess their relative contributions. The structural characteristics of a lesion which determines its excisability by individual excision pathways are expected to

be different. Excisability may vary for different portions of the genome and for chromatin subfractions such as the DNAse I or the staphylococcal nuclease digestible and resistant portions of chromosomal DNA (see below).

LESION PERSISTENCE: The fraction and amount of a specific lesion which remains in the genome after the excision rate has dropped below detectibility.

CODING PROPERTIES: The chemical structure of lesions which are present in the genome when they are reached by the replication- and transcription- machinery determines whether they are *instructive* or *non-instructive*[15]. Instructive lesions may code normally, i.e. possess the coding properties of the parent nucleotide from which they are derived or they may miscode. Non-instructive lesions have lost the ability to act as a template and may or may not act as a temporary or permanent block to replication and/or transcription. The presence of non-instructive lesions may result in the "perturbation of daughter strand synthesis" and in "perturbation of transcription".

REGULATORY PROPERTIES: DNA lesions per se or, DNA configurations formed as intermediates during lesion processing, may induce or modulate DNA-repair and DNA-synthesis functions. It may be necessary in the future to define additional biochemical properties of lesions, e.g. repairability by mechanisms which do not involve lesion excision, efficiency for the induction of sister chromatid exchanges and chromosomal aberrations, etc.

Ideally the biochemical properties of prototype DNA lesions should be defined in standard pro- and eu-karyotic cell systems in quantitative rather than qualitative terms since the situation will be rare where a lesion is not excised at all, miscodes a 100% of the time, is completely non-instructive, etc. Prokaryotic systems, in particular *E. coli* and its bacteriophages, are extremely useful for quantitative studies of this type. Analogous experiments with mammalian viruses are promising and have already yielded important results. The characterization of the biochemical properties of DNA lesions in intact mammalian cells is more difficult. However, progress is being made in the determination of the relative excisability and persistence of individual, chemically characterized lesions in intact mammalian cells and some of this work is summarized below.

"Excisability" is a raw measure of the efficiency of lesion removal in a particular cell which is defined because more precise enzymological terms cannot be applied to the intact cell. The term does not contain information about the

mechanism(s) of lesion excision or the subsequent steps of repair which accomplish reconstitution of the DNA strand. Excisability is measured by observing the disappearance of specific lesions or specific endonuclease sensitive sites from high molecular weight DNA and cannot, in general, be assessed by repair replication phenomena. The term is biologically meaningful if lesion removal is rate determining and if different excision repair pathways possess comparable fidelity. Excisability is determined by lesion properties and cellular properties. Important lesion properties are: chemical structure, effect on local DNA and chromatin conformation[16], intragenomic location(see below); Important cellular properties are: tissue and species, state of growth, state of differentiation.

The excisability of several structurally characterized lesions has been assessed in pro- and eukaryotic cells. I have chosen recent work with human cells as an example for our discussion. A simple relationship between the excisability of (presumably) pyrimidine photodimers and the toxicity and mutagenicity of far-ultraviolet light was observed by Maher and her collaborators [17,18] for the different complementation groups of *Xeroderma pigmentosum* (XP). An inverse relationship was found between residual excisability of photodimers and mutagenicity and lethality of equal doses of ultraviolet light. In analgous experiments the toxicity and mutagenicity of K-region epoxides of several polycyclic aromatic hydrocarbons and of N-acetoxy-AAF were found to be highest in the XP-strains with lowest residual excisability of far-ultraviolet lesions. However, the excisability of the lesions introduced by these ultimate chemical damaging agents was not determined and no direct correlation is possible, therefore. A similar relationship may exist between the sensitivity of *Ataxia-telangiectasia* skin fibroblasts and their capability to remove *Micrococcus luteus* γ-endonuclease sensitive sites[19] but no quantitative correlation has been possible so far.

In our laboratory we are investigating the relative excisability of prototype guanine lesions in human epitheloid lung cells A549[20] with the aim to relate excisability and toxicity and to define the structural properties of the lesions by which excisability is determined. This cell line is particularly useful since it has retained the capability to metabolize drugs such as polycyclic aromatic hydrocarbons in culture. The following arylation lesions of guanine have been studied: (1) The major products formed upon exposure of A549 to benzo(a)pyrene (B(a)P)[21] (7R) N^2-(10-{7β, 8α, 9α-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}deoxyguanosine (B(a)P-diol-epoxide I-dG) and (7R or 7S) N^2-(10-{7β,-

8α, 9β-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl)deoxyguanosine (B(a)P-diol-epoxide II-dG) (2) the guanine and possibly cytosine adducts formed upon exposure of A549 to the K-region epoxide 4,5-dihydrobenzo(a)pyrene-4,5-epoxide (B(a)P-4,5-epoxide C_1 and B(a)P-4,5-epoxide C_2) (3) the lesions formed upon reaction of A549 with N-acetoxy-AAF: "major lesion " 8-[N-(aminofluorenyl)]deoxyguanosine (AAF-(C_8)dG and its deacetylated derivative 8-[N-(aminofluorenyl)] deoxyguanosine (AF- (C_8)dG); "minor lesion" N^2-[3-(2-acetylaminofluorenyl)]deoxyguanosine (AAF-(N_2)dG)[22]. In all these guanine lesions bulky aryl-substituent have been introduced but the position of substitution and the size, stereochemistry and electronic properties of the polycyclic ring systems are different. In a first series of experiments each damaging agent was studied separately and in a second series pairs of damaging agents were studied in the same culture. Under the latter conditions effects of toxicity and cell growth on excisability are the same for the lesions which are being compared and lesion interaction can be investigated. The results of a typical experiment with B(a)P plus N-acetoxy-AAF are given in Figure 1 and 2. Figure 1 shows the chromatographic profiles of DNA digests of A549 cultures which were first treated with [^{3}H]B(a)P for 48 hours, then with [^{14}C]-N-acetoxy-AAF for 45 min. and subsequently incubated for 0 and 21 hours. The initial levels of damage and the amount of each individual lesion removed as a function of post-treatment incubation is plotted in Figure 2 (it was not possible to measure DNA-acetylation in our experiment). From experiments of this type the following scale of excisability of arylation lesions of guanine was derived: (The values listed above the lesions represent the fractions of lesions removed within one generation time under non-saturating conditions. Relevant data from other investigators obtained with different human cells is included for comparison [23-25]).

$$\underset{\text{B(a)P-4,5-epoxide } C_2}{\overset{1.0}{}} > \underset{\text{B(a)P-4,5-epoxide } C_1}{\overset{0.80}{}} >> \underset{\text{B(a)P-diol-epoxide I dG}}{\overset{0.42}{}} \geq \underset{\text{B(a)P B}_0}{\overset{0.24}{}} \geq \underset{\text{AAF - (}C_8\text{)dG}}{\overset{0.22}{}} \geq \underset{\text{AAF - (}N_2\text{)dG}}{\overset{0.18}{}}$$

$$\underset{\text{epoxide II dG}}{\overset{0.58}{}} > \underset{\text{AF - (}C_8\text{)dG}}{\overset{0.36}{}} > \overset{0.17 - 0.32}{}$$

Skin	AAF-dG	0.23 - 0.40[23]
HeLa	BrMeBA - dG	0.26[24]
Lymph. Raji	AAF - dG	1.0 (?)[25]

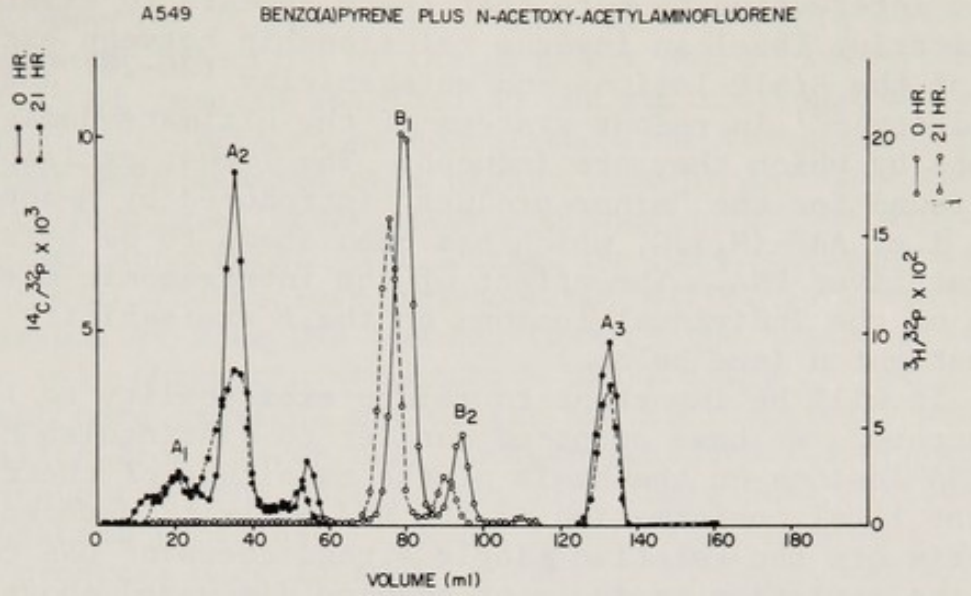

Fig. 1 Sephadex LH-20 chromatography of enzymatic digests of DNA from A549 cells which were prelabeled with $^{32}PO_4^{-2}$ grown for 48 hrs. in the presence of 1.3μM [^{3}H]benzo(a)pyrene and then treated with 6μM [^{14}C]N-acetoxy-acetylaminofluorene and incubated for 0 or 21 hrs. Ratios of the ^{14}C and ^{3}H radioactivity in each fraction over total ^{32}P radioactivity contained in the DNA digest are plotted in order to correct for DNA damage dilution due to cell growth. A_1, "minor" AAF lesion, AAF-(N_2)dG; A_2, "major" AAF lesion AAF-(C_8)dG; A_3 (probably) deacetylated "major" AAF lesion AF-(C_8)dG; B_1, B(a)P-diol-epoxide I-dG; B_2, B(a)P-diol-epoxide II-dG.

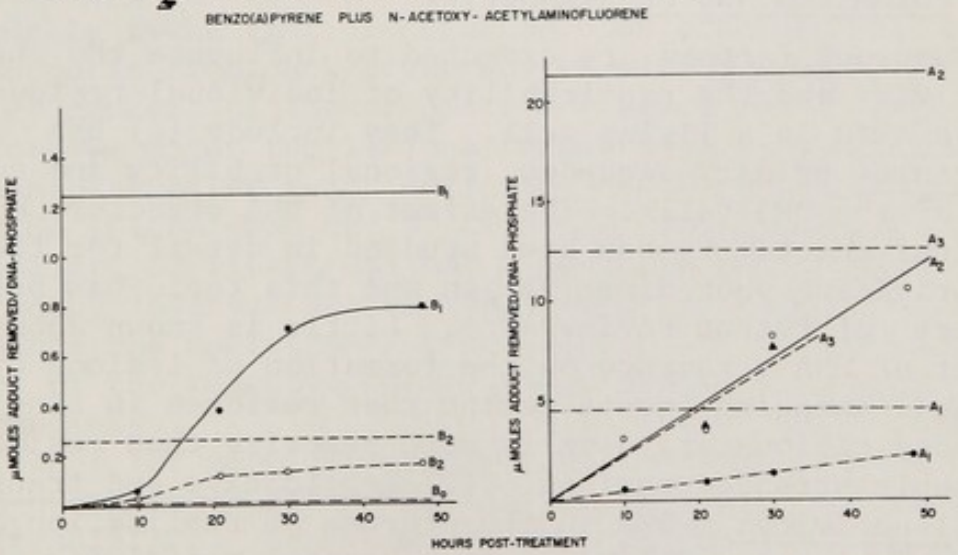

Fig. 2. Kinetics of excision from DNA of benzo(a)pyrene and N-acetoxy-acetylaminofluorene lesions in A549 cells. Data was derived from experiments of the type shown in Fig. 1 and the same symbols are used for the lesions. The horizontal lines mark the level of the individual lesions at the outset of post-treatment incubation. Left side: B(a)P lesions (lesion B_0 has not been chemically identified); right side: AAF lesions.

It is interesting to note that there appears to exist on a qualitative level an inverse relationship between excisability of the B(a)P lesions and mutagenicity [26-28] and carcinogenicity[29] in rodent systems of the ultimate damaging agents by which they are induced. The lowest excisability was found for the "minor product" introduced by N-acetoxy-AAF, i.e. AAF-(N_2)dG, which has been shown to be persistent in rat liver DNA. The effect of the intragenomic distribution of the individual lesions on their excisability is being investigated (see below.)

It will be important to relate excisability to lesion structure. We have proposed earlier to distinguish classes of DNA lesions on the basis of the magnitude of their effect on the local conformation of the DNA helix.[16] In recent experiments the relative single strandedness of DNA containing the arylation products of guanine discussed above was determined by comparing the susceptibility of the damaged DNA to digestion by single-strand specific S_1-nuclease[30-32]. In general it appears that lesions inducing extensive local single-strandedness also possessed relatively high excisability in A549 cells. These findings point out the potential usefulness of a classification of DNA lesions on the basis of local helix distortion[16] *but they do not prove that helix distortion is the only or the most important lesion property which determines excisability.*

(3) EFFECT OF DNA AND CHROMATIN STRUCTURE ON LESION FORMATION AND REPAIRABILITY

Numerous factors are expected to influence the chemical reactivity and the repairability of individual residues in DNA *in situ* in a living cell. They include (1) DNA structure: primary sequence, regional stability and conformation of the DNA helix. The effect of DNA structure on its chemical reactivity has been studied in detail for the case of pyrimidine photodimerization and this topic has been subject of recent reviews[33]. Little is known about the effect of DNA structure on the formation of lesions by chemical damaging agents except that residues in single stranded regions are usually more reactive than residues in double stranded regions. (2) Replicative and transcriptional activity: DNA in the process of replication or transcription may differ in its chemical reactivity and repairability from "inactive" DNA. Evidence for increased repair activity in DNA growing points relative to bulk DNA has been obtained in human lymphoblastoid cells following treatment with N-methyl-N'-nitro-N-nitrosoguanidine and methylnitrosourea[34]. Preferential introduction of 2-acetyl-aminofluorene (AAF) into the DNA portions which were highly sensitive to pancreatic DNAse I (at 8-10% solubiliza-

tion) was observed in rat liver within 10 to 30 min. following intraperetoneal injection of the drug but not at later times[35]. On the basis of results obtained in the reticulocyte and oviduct system[36,37] it can be speculated that transcriptionally active genes were damaged at a higher rate by AAF than the rest of the DNA immediately after application of the drug. (3). Nuclear structure: DNA located on the nuclear periphery in contact with the nuclear membrane may differ in reactivity and repairability from DNA located towards the center of the nucleus. (4) Chromatin structure: DNA located in the nucleosomal core particle may differ in its reactivity and repairability from linker DNA; further reactivity differences may originate from the interaction with individual histones and non-histone proteins. The DNA repair field rapidly exploits progress made in basic molecular biology. It will be interesting to employ any new technology which allows the separation of structurally or functionally distinct chromatin regions for the study of damage formation and repair.

Recent progress in the understanding of chromatin structure and in particular the development of simple procedures which distinguish between nucleosomal-core and -linker DNA on the basis of their resistance and susceptibility to digestion by staphylococcal nuclease allow a first look at the effect of chromatin structure on lesion distribution and repairability. In experiments with highly reactive ultimate chemical damaging agents the nucleosomal linker DNA was more reactive than core DNA. The damage level of linker DNA was approximately twice that of core DNA upon treatment of isolated chromatin from duck erythrocytes with N-acetoxy-AAF[38]. The adduct content of linker DNA was 4 times and 11 times higher for the major two lesions formed by the reaction of intact human alveolar tumor cells A549 with benzo(a)pyrene-4,5-epoxide (G. Feldman and P. Cerutti, unpublished). Higher susceptibility of linker DNA may also be found for damage introduced by indirect action of chemical and physical agents since the nucleosomal proteins may act as efficient quenchers of the reactive oxygen species which are formed as intermediates.

Evidence for more rapid repair of lesions in linker DNA has been obtained in human skin fibroblasts following ultraviolet irradiation. Immediately after irradiation repair replication occurred preferentially in staphylococcal nuclease digestible linker DNA[39,40]. Similar results were obtained following treatment of mouse mammary gland with methylmethanesulfonate.[41] A higher initial level of damage in the linker DNA may in part be responsible for these results, especially for the studies using methylmethanesulfonate. Evidence for non-random distribution of alkylation damage has been obtained earlier[34].

The relative rate of the following processes influence the intragenomic distribution of the lesions for damaging agents which require metabolic activation (1)rate of formation of ultimate damaging agent(s) (2) relative rates of lesion formation and removal in different parts of chromatin. The rates of these processes and therefore the level of lesions and their distribution in DNA are expected to undergo continued changes following administration of the drug. Experiments with the liver procarcinogens dimethylnitrosamine (DMN) and AAF are in agreement with this expectation. The intragenomic distribution of methylated bases and AAF-adducts and their removal from DNA was measured in rat liver. In both cases the damage level was considerably higher in the nuclease digestible portions of chromatin soon after drug treatment. The damage levels decreased and became more comparable in the digestible and resistant DNA fractions when the post-treatment period was extended over several days[35,42]. A similar result was obtained when human alveolar tumor cells A549 were treated with N-acetoxy-AAF (J. Remsen and P. Cerutti, unpublished). Since staphylococcal nuclease was used in the studies with AAF it can be concluded that the formation and removal of AAF-adducts was more efficient in the nucleosomal linker DNA. No attempt was made to distinguish individual lesions in this work. However, there is no reason to expect equal patterns of distribution for all lesions introduced by a given damaging agent. This question is of particular importance since the excisability and persistence of individual lesions are likely to be influenced by their location in the genome. Equal distribution of B(a)P-diol-epoxide II-dG between nucleosomal core- and linker- DNA was observed in actively B(a)P metabolizing A549 cells while the level of B(a)P-diol-epoxide I-dG was slightly higher in the core-DNA. The excisability of the two adducts was the same in core- and linker DNA during post-treatment incubation up to 34 hours (J. Remsen, G. Feldman and P. Cerutti, unpublished).

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Workshop Summary: DISTRIBUTION AND QUANTITATION OF DNA DAMAGE

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One of the most persistent problems encountered in the study of DNA repair has been the quantification of various DNA lesions at levels which are of biological significance. Most chemical and even radiochemical procedures are not sufficiently sensitive. Recently, assays using specific enzymes have been developed which show the necessary sensitivity. However, the accuracy of these assays depends upon knowing what type of damage is recognized by the enzyme used. Obtaining this information has been complicated by the fact that most DNA damaging agents - UV, X-radiation, alkylating agents, for example - produce several types of lesions, and in different proportions. The disparity in the sensitivity of chemical and enzymatic assays often makes it difficult to identify which of the lesions an enzyme considers its proper substrate. The situation is further complicated by the apparent diversity of enzymes with overlapping but non-identical specificities. In spite of these difficulties, however, the sensitivity and relative simplicity of enzymatic assays is resulting in increasingly wider use (see 1 for review).

The prototype lesion for developing the enzymatic assays was the pyrimidine dimer. Two endonucleases specific for dimers, one from *Micrococcus luteus* and one from bacteriophage T4, have been used to determine the pyrimidine dimer content of UV-irradiated DNA. The assays measure the number of nicks per DNA molecule resulting from endonuclease treatment. Their sensitivity, therefore, depends upon the molecular weight of the untreated control DNA. Typically this has been $1\text{-}2 \times 10^8$ in studies of DNA repair in bacterial or mammalian cells. With DNA of this size the number of pyrimidine dimers produced by 1-2 J/m^2 at 254 nm can easily be measured in a sample of 100 ng of DNA. This corresponds to approximately 2 dimers per 10^8 daltons, or 10^{-17} μmoles of pyrimidine dimers per 10^6 DNA phosphates. Recently developed methods which permit measuring 1-2 nicks per 10^9 (2, 3, 4) daltons should increase the sensitivity of the assays by a factor of 10.

Two procedures have generally been used to quantify nicks for these assays. Linear DNA has been sedimented in alkaline sucrose gradients and the number of nicks produced by endonuclease treatment calculated from number average molecular weights (5). Alternatively, *in vitro* studies have often utilized superhelical DNA as substrate. In this case the conversion of supercoils to nicked circles has been determined using sedimentation, electrophoresis or binding to nitrocellulose filters to separate nicked from unnicked molecules. The aver-

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age number of nicks per molecule has been calculated by the Poisson expression from the proportion of molecules remaining unnicked. The procedures should give similar results although the underlying principles are different. However, data presented in this workshop indicated that the apparent yield of nicks produced by the pyrimidine dimer specific T4 endonuclease V as a function of UV dose to the substrate DNA was two-fold higher for superhelical than for linear DNA (Ganesan, Friedberg and Seawell, unpublished results). The discrepancy appeared to be due to the method of analysis, not to a difference in the response of superhelical and linear DNA to UV irradiation or to T4 endonuclease V. This conclusion was based upon studies of colE1 DNA treated with the restriction endonuclease EcoRI to produce unit length linear molecules. When analysed on alkaline sucrose gradients UV irradiated DNA treated with T4 endonuclease V appeared to contain the same number of nicks whether EcoRI treatment occurred before irradiation, after irradiation or after T4 endonuclease V treatment. The cause of the discrepancy is not known, but may reflect a slightly non-random distribution of pyrimidine dimers in irradiated DNA (cf. Grossman, this volume).

Enzymatic assays have primarily been used to detect pyrimidine dimers but they can, in principle, be extended to any lesion for which a specific endonuclease or DNA glycosylase can be found. Although glycosylases do not nick DNA they produce apurinic or apyrimidinic (AP) sites which will yield nicks upon treatment with alkali or AP endonuclease. Some enzymes of actual or potential interest for detecting damage include AP endonucleases (6, 7), 3-methyl adenine glycosylase (8), uracil glycosylase (9, 10) and endonucleases active against unknown lesions in DNA treated with γ- or X-rays (11; Wallace, et al., this volume), osmium tetroxide (12) or acetylaminofluorene (13).

In order to use an enzyme for measuring DNA damage it is desirable to know precisely what type of lesion the enzyme recognizes. Two of the speakers in this workshop drew attention to difficulties encountered in determining the type of lesion recognized by an enzyme when the substrate DNA contains more than one kind of damage and the enzyme preparation being examined contains more than one activity. These difficulties were exemplified by experiments with enzymes which attack "alkali labile" sites in DNA exposed to alkylating agents or ionizing radiation (Brent, et al., this volume; Wallace, et al., this volume). Alkali labile sites in this context are lesions which will yield DNA strand breaks under alkaline but not under neutral conditions. Two types have recently been distinguished: 1) those sites sensitive to treatment with 0.1 N NaOH for 20 minutes at 37°C ; 2) those requiring more extensive treatment, e.g. 4 hours at room temperature in

1 M glycine-NaOH (pH 12.8). According to Teebor, et al (this volume) AP sites belong to the first category, and acid heat depurinated DNA contains only this type of site. However, DNA exposed to X-radiation in the presence of KI, alkylated with MMS or irradiated with high doses of UV contain both types. They are not AP sites, but they may be altered bases which can dissociate to leave AP sites. 3-methyl adenine may belong in this category. Currently the only way to distinguish the two types of alkali labile site is to specify the treatment required to convert them to strand breaks. The distinction is important for characterizing enzyme preparations. Purified AP endonucleases attack only the first type of site. However, other enzyme preparations will attack both types. In some cases this results from the presence of more than one enzyme (8; Brent, et al., this volume), while in other cases a single endonuclease appears to be responsible (Wallace, et al., this volume).

Several laboratories are currently purifying and characterizing enzymes involved in DNA repair. As knowledge of individual biochemical reactions contributing to repair increases so will the number of enzymes available for measuring DNA damage and the variety of lesions which can be measured enzymatically. At the present time, however, the characterization of enzymes and the identification of lesions produced by various physical and chemical agents are interdependent investigations. Frequently the first evidence for an erstwhile unrecognized enzyme is its effect upon a previously unsuspected lesion. Since many of the enzyme preparations now being used to monitor DNA damage are not highly purified and contain activities other than the one of primary interest they may yield exaggerated estimates of a particular type of damage. It is essential to remember this limitation when interpreting results of enzymatic assays.

Illustrating a different approach to quantifying DNA damage Rahn, et al. (this volume) described a model system using iodinated cytosine. DNA containing 5-iodo cytosine (IoCyt) can be produced *in vivo* by growing bacteria with IoCyt, and *in vitro* by a chemical reaction of iodide with single stranded DNA (14, 15). Irradiation of DNA containing iodinated bases with 313 nm light causes selective loss of iodine, which can be monitored using ^{125}I. Two potentially interesting applications of this system were indicated. 1) The types of lesion produced by photolysis should be limited in number and therefore relatively easy to characterize and quantify; 2) the chemical iodination reaction strongly prefers cytosine in single stranded DNA (15) and may provide a probe for damage caused by agents which act on guanine and disrupt hydrogen bonds.

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LESIONS IN ALKYLATED DNA DETERMINED BY SUSCEPTIBILITY TO ALKALI, APURINIC ENDONUCLEASE OR N-GLYCOSIDASE[1]

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ABSTRACT To detect and quantitate lesions in methyl methanesulfonate-treated DNA, we converted these sites to strand breaks by 5 different parallel procedures. Apurinic sites alone were so converted by apurinic endonuclease from human CEM-CCRF lymphoblasts or by a 20-min treatment with 0.1 M NaOH. Lesions in addition to these were converted by whole-cell extracts (CEM-CCRF or HeLa), by apurinic endonuclease plus 3-methyladenine N-glycosidase (both from CEM-CCRF), or by a 4-hr treatment with 1.0 M glycine-NaOH.

INTRODUCTION

The simplest way to determine physically or chemically induced lesions in DNA is to degrade the DNA and isolate the altered moiety. When this is not feasible, primary lesions may be converted to more readily measured secondary forms. In the case of methyl methanesulfonate-treated DNA (MMS-DNA), enzyme activities from human cells convert some damage to strand breaks and may be useful probes for specific lesions. However, reports of the susceptibility of MMS-DNA to damage-specific enzymes from human cells are in apparent conflict.

Duker and Teebor (1) reported that maximum strand breakage following treatment with HeLa-cell sonicates was equal to that following alkaline treatment by the method of Lindahl and Andersson (2). Because alkali-labile sites, determined by the latter method, had been equated with apurinic sites in assays with heat-depurinated DNA (2), the conclusion was that human-cell extract acted only on apurinic sites in MMS-DNA. By

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contrast, Brent (3) reported that lesions in addition to apurinic sites were sensitive to N-glycosidase activity, isolated from CEM-CCRF lymphoblasts. In this study, however, the methods for quantitating apurinic sites differed from those used by Duker and Teebor. Hence, discrepancies between the studies might be related to the specificity of the procedures used to determine apurinic sites. We now report a comparative study of procedures, including the ones used in these two studies, for determining alkali-labile and enzyme-sensitive sites in MMS-DNA.

METHODS

DNA Treatments. PM2 viral DNA was either heat-depurinated at 70°C, pH 5.5 (4), or alkylated with MMS (12.5×10^{-3}M) at 37°C for 5, 10, or 30 min and re-isolated by gel filtration (1).

Assays of Alkali-Labile Sites. Method A: DNA was incubated in 1.0 M glycine-NaOH for 4 hr at room temperature (2). Method B: DNA was incubated in 0.1 M NaOH for 20 min at 37°C (5,6). In both methods, the prevalence of strand breaks was estimated from the proportion of nicked DNA, determined by alkaline-CsCl sedimentation analysis (4).

Assays of Enzyme-Sensitive Sites. Alkylated PM2 DNA was diluted to 0.3-0.5 µg/100 µl in 0.5 mM dithiothreitol, 0.06 M Hepes-KOH (pH 8.0), and 0.6 mM $MgCl_2$ prior to addition of 5 µl of apurinic endonuclease purified from human CEM-CCRF lymphoblasts (5). In some assays, 3 µl of N-glycosidase from CEM-CCRF cells was also added (3).

For assays with crude-cell extracts, 20 µl of HeLa-cell sonicate or 10 µl of CEM-CCRF cell homogenate (in 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 0.5 mM dithiothreitol) was added to 10 µl of 1.0 M Tris-phosphate (pH 7.0), 1 mM EDTA. The final reaction volume was 120 µl.

All enzyme reactions were for 30 min at 37°C. The extent of strand breakage was estimated from the proportion of nicked DNA, determined by neutral-CsCl sedimentation analysis (4). Background values (for strand breaks in DNA prior to assay) have been subtracted for all reported data.

RESULTS AND DISCUSSION

When heat-depurinated PM2 DNA was treated with alkali according to methods A or B, comparable numbers of strand breaks were produced in each case (7). Consistent with previous reports (4,5,8), these values also corresponded closely with the number of apurinic sites converted to strand breaks by either crude extract from HeLa cells or by purified apurinic endonuclease from CEM-CCRF lymphoblasts.

By contrast to these results for heat-depurinated DNA, discrepancies occurred among results for these same methods when MMS-DNA was analyzed. Table 1 shows that alkaline hydrolysis by method A gave about three times as many strand breaks as did method B. Assays with purified apurinic endonuclease, a specific probe for apurinic sites, gave values comparable to those of method B for alkali-labile sites. We have estimated from the data reported by Ljungquist et al. (9) that their MMS-DNA also had about 3 alkali-labile sites (determined by method A) for every 1 site that was sensitive to purified apurinic endonuclease from calf thymus---consistent with our findings.

Our interpretation of these data is that method B allows the accurate determination of apurinic sites that exist immediately after MMS treatment, while method A appears to promote the hydrolysis of additional sites, possibly alkylated bases or phosphotriesters (6). The contention that alkylated DNA is stable at high pH (9) does not appear to hold for the conditions of method A.

In further comparisons, the amount of strand-breakage caused by crude extracts was over twice that by purified apurinic endonuclease (Table 1). However, with the addition of

TABLE 1
ALKALI- AND ENZYME-SENSITIVE SITES IN MMS-TREATED DNA

Reaction time with MMS	Alkali-labile sites		Enzyme-sensitive sites		
	method A	method B	AP endo[a]	crude extract[b]	AP endo+ N-glyco[c]
5 min	0.15[d]	---	0.04	0.28*	---
	0.18	---	0.06	0.28	---
10 min	0.85	0.12	---	---	---
	1.0	0.12	---	---	---
30 min	1.8	0.51	0.55	1.3 **	1.2
	1.8	0.63	0.65	---	1.5
30 min	1.3	0.48	---	---	---
	1.5	0.52	---	---	---
30 min	1.6	---	0.64	1.5 *	0.87
	1.9	---	0.72	1.5	1.1
30 min	---	0.45	---	---	---
	---	0.48	---	---	---

[a]Purified apurinic endonuclease. [b]From HeLa cells (*) or CEM-CCRF cells (**). [c]Purified apurinic endonuclease combined with partially purified N-glycosidase. [d]Each value is the average number of strand breaks per DNA molecule.

N-glycosidase to apurinic endonuclease, strand breakage approached that of crude extracts. These findings suggest that crude extract attacks more than one kind of lesion: apurinic sites (sensitive to endonuclease) and alkylated bases (sensitive to N-glycosidase plus endonuclease). Supporting evidence for this includes a recent report by Laval (10) that 3-methyladenine N-glycosidase combined with apurinic endonuclease (both purified from M. luteus) produces strand breakage at non-apurinic sites in MMS-DNA. Similar to the bacterial enzyme, the human N-glycosidase that we used releases 3-methyladenine but not 7-methylguanine, and thus, may be a suitable probe for the former in alkylated DNA.

In conclusion, our findings emphasize the need to establish the specificity and stoichiometry of various methods for converting primary lesions to secondary forms. While all of the procedures that we assessed are quantitative for apurinic sites in heat-depurinated DNA, only those done with purified apurinic endonuclease or alkaline hydrolysis by the methods of Brent (5) or Shooter and Merrifield (6) are specific for these sites in MMS-DNA. Whole-cell extracts, on the other hand, provide a measure of the sum total of enzyme-sensitive damage, while the Lindahl-Andersson method of alkaline hydrolysis (2) may detect a broader spectrum of damage.

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ENZYMATIC RECOGNITION OF DNA DAMAGES INDUCED BY IONIZING RADIATION[1]

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ABSTRACT Both alkali-labile and alkali-stable damages in x-irradiated PM2 DNA are recognized by purified fractions of the Escherichia coli x-ray endonuclease. An activity, partially purified from Saccharomyces cerevisiae using an x-irradiated PM2 DNA substrate, appears only to recognize alkali-labile lesions.

INTRODUCTION

An enzyme activity partially purified from E. coli on the basis of its ability to nick x-irradiated PM2 DNA (1,2), has been shown to recognize alkali-labile and alkali-stable damages in such DNA (2-4), as well as DNA damages induced by osmium tetroxide (3). A similar activity has also been demonstrated in Micrococcus luteus extracts (5), and has recently been resolved into five fractions (6). Apurinic endonucleases isolated from E. coli (7,8), and other sources (for a review see 9), also nick x-irradiated DNA presumably at apurinic/apyrimidinic sites.

In this study, we report the specificity of purified fractions of the E. coli x-ray endonuclease as well that of partially purified Saccharomyces cerevisiae (yeast) apurinic activities.

RESULTS AND DISCUSSION

The E. coli x-ray endonuclease has been further purified by DNA agarose affinity chromatography and phosphocellulose ion exchange chromatography. In 10 mM Tris, pH8, the enzyme elutes at 0.6M KCl on DNA agarose; in 10 mM $MKPO_4$, pH 6.5 it elutes at 0.5 M KCl on phosphocellulose. By using these methods, 5000 fold purification is achieved with small scale preparations, while 1000-2000 fold purification is achieved with larg-

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er-scale preparations. Fraction IV exhibits the same specificity on x-irradiated PM2 DNA substrate as does less purified fractions or crude extracts. That is, with DNA x-irradiated in the presence of the hydroxyl radical scavanger potassium iodide (KI), the enzyme recognizes fast-converting alkali-labile lesions (204), slow converting alkali-labile lesions (4), and alkali-stable damages (2-4). Strand breaks, whether radiation-induced, alkali-induced, or enzyme-induced, are measured by the conversion of PM2 Type I DNA to Type II using sucrose gradient sedimentation analysis (1-4). E. coli x-ray endonuclease Fraction IV also nicks osmium tetroxide-treated DNA, heat depurinated DNA and ultraviolet-irradiated DNA containing 180 pyrimidine dimers per PM2 molecule. Purified

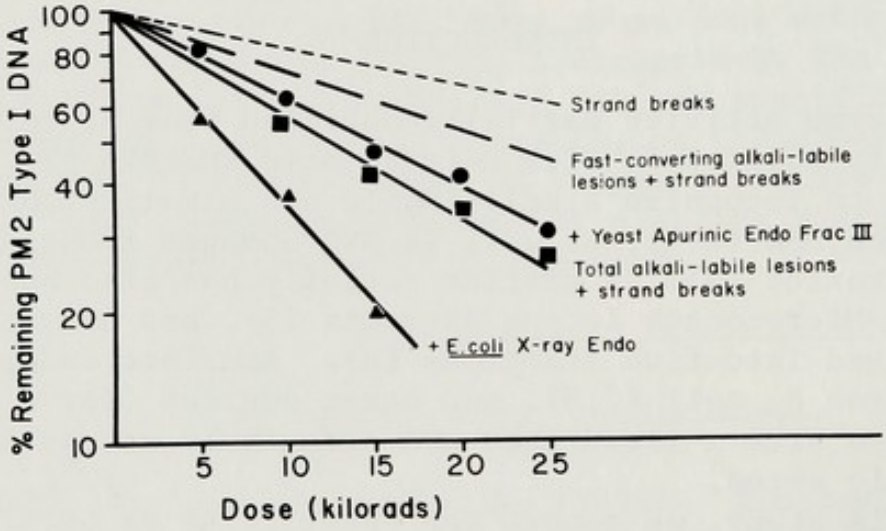

Figure 1. Analysis of strand breaks in x-irradiated PM2 DNA. ----, x-ray-induced single-strand breaks determined by neutral sucrose gradient analysis (1-3); —— ——, x-ray induced alkali-labile lesions analyzed directly on alkaline sucrose gradients (2-4); ■ , total alkali-labile lesions determined after 16 hours incubation in 1M glycine buffer, pH 12.8 (10) before alkaline sucrose gradient analysis (4). The same line is generated if the DNA is incubated with yeast apurinic enzyme Fraction III before analysis; ● , x-ray-induced single-strand breaks plus x-ray-induced damages recognized by Fraction III yeast apurinic enzyme as analyzed on neutral sucrose gradients; ▲ , x-ray-induced single strand breaks plus x-ray-induced lesions recognized by the E. coli x-ray endonuclease as analyzed on neutral sucrose gradients (2,3). The same line is generated if the DNA is analyzed by alkaline sucrose gradients after incubation in glycine buffer (4).

TABLE 1
PROPERTIES OF DNA-AGAROSE FRACTIONS OF YEAST ENDONUCLEASE(S)

Fraction Number	ENZYME-BREAKS / PM2DNA MOLECULE / 30 MIN					
	Per Krad X-Irradiated DNA	Per Apurinic Site			UV-Irrad. DNA 30 Dimers Molecule	Unirrad DNA
	+Mg++[a] +ATP [b]	+Mg++ +ATP	-Mg++ -ATP	+Mg++ +ATP +ETDA[c]	+Mg++ +ATP	+Mg++ +ATP
I	0.021	0.170	-	0.066	0.130	0.08
II	0.027	0.609	-	0.208	0.200	0
		0.257	0.218	0.157		
III	0.022	0.787	0.900	0.773		
	0.025	0.946	-	0.607	0.080	0
		0.748	0.640	-		

[a] 8mM $MgCl_2$; [b] 2mM ATP; [c] 8mM EDTA

fractions do not exhibit any activity on unirradiated DNA either in the presence or absence of magnesium; and after DNA-agarose chromatography, it is not necessary to add calf thymus DNA to compete for non-specific nucleases. As with crude preparations, purified E. coli x-ray endonuclease does not require magnesium, has a broad pH optimum (6.5-8.5) and is fully active in the presence of 10mM EDTA.

An activity which nicks x-irradiated PM2 DNA has also been demonstrated in crude extracts of yeast. This activity has been resolved on DNA agarose columns into three Fractions which have subsequently been shown to have apurinic activity. Some properties of these three activities from preliminary data are summarized in Table 1. Fraction III, which elutes at 0.6 M KCl in 20 mM KPO_4, pH8, is roughly 100 fold purified. Under the reaction conditions used, Fraction III nicks about 79% of the alkali-labile sites in heat depurinated DNA and 72% of the total alkali-labile lesions in DNA x-irradiated in the presence of KI (Figure 1). Fraction III does not appear, from quantitative considerations, to recognize alkali-stable lesions in x-irradiated DNA, and thus seems to be an apurinic enzyme. Further studies on the specificity of this enzyme are currently in progress.

The ability of both the E. coli x-ray endonuclease and the yeast apurinic enzyme to bind very tightly to DNA agarose

makes them good candidates for large scale purification and use as reagents. If the enzyme-induced breaks introduced into x-irradiated DNA are assayed using the *E. coli* x-ray endonuclease and the yeast apurinic enzyme, then an estimate of both endonuclease-sensitive alkali-labile and alkali-stable damages can be readily ascertained under neutral conditions. As Figure I shows, the use of the sensitive strand break assay on x-irradiated PM2 DNA combined with enzyme probes enables one to potentially quantify various classes of DNA lesions induced by x-rays *in vitro*. Extension of these studies to DNA irradiated *in vivo* is now in progress.

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Micrococcus luteus UV-ENDONUCLEASE-SENSITIVE SITES IN FAR- AND NEAR-UV-IRRADIATED CHINESE HAMSTER OVARY CELLS

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ABSTRACT *Micrococcus luteus*, UV-endonuclease-sensitive sites are induced in the DNA of Chinese hamster ovary cells both by far- and by near-UV radiations. Greater than 95% of the sites induced by either radiation source were susceptible to *in vitro*, enzymatic photoreactivation. The numbers of sites detected decrease upon incubation of cells such that 30% and 18% fewer sites were found 24 h after irradiation of the cells with 1.2 and 2.9 J/m^2 at 254 nm, respectively. Similar results were found for near-UV-induced, UV-endonuclease-sensitive sites.

INTRODUCTION

Low-pressure-mercury, germicidal lamps are common laboratory sources of far-ultraviolet (UV) radiation. Their emission spectra are relatively monochromatic with most of their radiant energy being emitted at 254 nm. However, many biological phenomena are strongly wavelength dependent and wavelengths below 290 nm are not frequently encountered outside of laboratories.

Cyclobutyl pyrimidine dimers are the predominant far-UV-induced DNA photoproducts *in vivo* and are biologically significant lesions in a wide variety of organisms. Their induction and fate in near-UV-irradiated cells is therefore of particular interest. Dimers are detected most directly by chromatographic procedures following the acidic hydrolysis of DNA, but such procedures are relatively insensitive at low doses. A more sensitive approach uses endonucleolytic activities from *Micrococcus luteus* to make single-strand incisions near pyrimidine dimers (1). We have investigated the suitability of a similar technique for the detection of pyrimidine dimers in near-UV-irradiated Chinese hamster ovary (CHO) cells, and have used this technique to examine dimer excision in CHO cells.

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MATERIALS AND METHODS

CHO cells, obtained from Dr. R. Humphrey, were grown as monolayer cultures at 37°C in Ham's F-10 medium (Flow) containing 15% fetal calf serum (Flow). This medium was supplemented with 0.25 μCi/ml [methyl-^{3}H] thymidine (Amersham, spec. act. 17 Ci/mmole) to prelabel cellular DNA.

Far-UV radiation was provided by a low-pressure-mercury, germicidal lamp (Philips, TUV) at an incident dose rate of 0.39 J/m^2/sec. Broad-spectrum, near-UV radiation was obtained from two "Sunlight" fluorescent lamps (Westinghouse) and was filtered through the lids of 60 mm plastic petri dishes (Greiner) to eliminate wavelengths below 290 nm.

UV-endonuclease-sensitive sites were determined by a procedure modified from that of Paterson, *et al.* (1). UV-endonuclease was prepared from *M. luteus* according to the procedure of Carrier and Setlow (2), and a preparation corresponding to their fraction II was used.

Photoreactivating enzyme purified from *Streptomyces griseus* was the generous gift of Dr. Eker. White fluorescent lamps were used as sources of photoreactivating light.

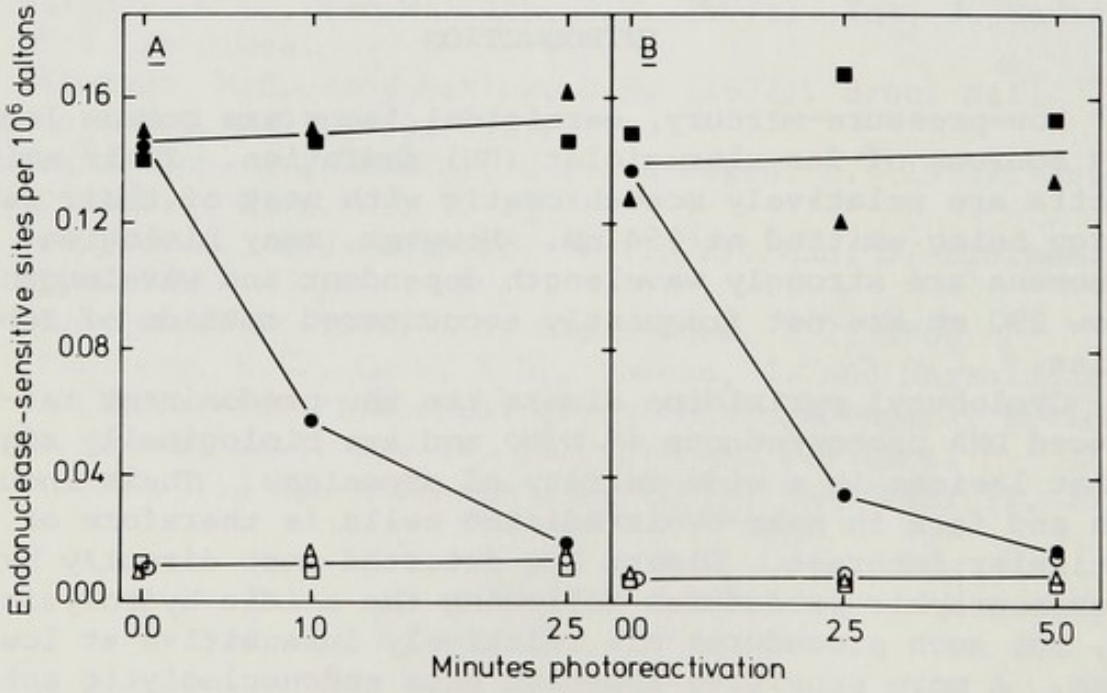

FIGURE 1. *In vitro*, enzymatic photoreactivation of ESS. Phenol extracted DNA was from unirradiated (open symbols), far-UV-irradiated (panel A, closed symbols), or near-UV-irradiated (panel B, closed symbols) CHO cells. The DNA was then incubated at 37°C with or without photoreactivating enzyme (PRE) and with or without exposure to white fluorescent light (PRL) prior to reextraction with phenol and ESS determination. +PRE, -PRL (Δ, ▲); -PRE, +PRL (□, ■); +PRE, +PRL (○, ●). Far-UV dose = 4.7 J/m^2.

RESULTS

M. luteus UV-endonuclease-sensitive sites (ESS) are induced in CHO cells both by far- and near-UV radiations. That these sites are related to the presence of pyrimidine dimers is indicated by their susceptibility to *in vitro*, enzymatic photoreactivation. More than 95% of the sites induced either by far- (Fig. 1, panel A) or by near- (Fig. 1, panel B) UV radiations are rendered insensitive to UV-endonucleolytic incision by prior exposure to photoreactivating enzyme (PRE) from *Streptomyces griseus*. Incubation of irradiated DNA with PRE in the dark or exposure to white fluorescent light in the absence of PRE had no effect of the numbers of ESS detected.

The fate of ESS in CHO cells incubated in medium at 37°C after irradiation is depicted in Fig. 2. Far-UV doses corresponded to 1.2 (panel A) and 2.9 (panel B) J/m^2. Near-UV exposures were chosen so as to induce numbers of ESS comparable

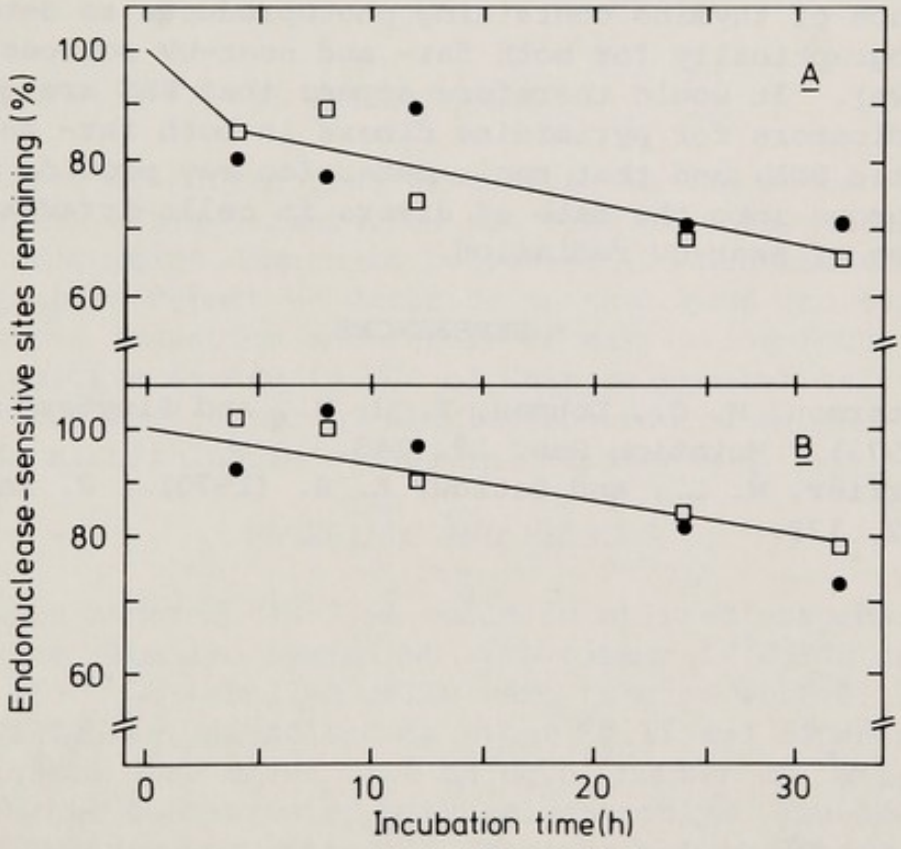

FIGURE 2. UV-endonuclease-sensitive site loss in CHO cells irradiated with far- (●) or with near- (□) UV radiations. Far-UV doses were 1.2 (panel A) and 2.9 (panel B) J/m^2, and near-UV exposures were chosen so as to result in the induction of comparable numbers of ESS. Near-UV exposure times were 40 and 100 sec in panels A and B, respectively. Data points are averages of three independent determinations.

to those induced in the far-UV-irradiated cells. After all irradiations a slow loss of ESS was found during postirradiation incubations of CHO cells, with greater proportions of the ESS being lost after lower doses. Approximately 30% and 18% of the total sites induced were lost during the first 24 h after irradiation with far-UV doses of 1.2 and 2.9 J/m^2, respectively. The rates of site loss in near-UV-irradiated cells were indistinguishable from those in far-UV-irradiated cells after the induction of comparable numbers of ESS.

DISCUSSION

Both far- and near-UV radiations induce ESS in CHO cells as linear functions of dose (data not shown). The photoreactivability of those sites demonstrates the specificity of the *M. luteus* extract for pyrimidine dimers in both far- and near-UV-irradiated DNA's. Furthermore, the dose dependence of ESS induction was found to be in reasonable agreement with the dose dependence of thymine containing photoproducts as determined chromatographically for both far- and near-UV sources (data not shown). It would therefore appear that ESS are quantitative indicators for pyrimidine dimers in both far- and near-UV-irradiated DNA, and that their detection may provide a sensitive probe into the fate of dimers in cells irradiated with low doses of near-UV radiation.

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REPAIR OF ALKYLATED DNA IN CHINESE HAMSTER CELLS MEASURED BY LOSS OF ENZYME-SENSITIVE SITES IN ISOLATED DNA.

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ABSTRACT Lesions introduced into the DNA of Chinese hamster cells by dimethyl sulphate (DMS) are recognized by an enzyme (s) present in crude extracts of M. luteus. Such lesions are rapidly lost from DNA during post treatment incubation of cells. The number of these enzyme-sensitive sites in methylated DNA, their rate of loss and their heat lability are consistent with the majority being 3-methyladenine adducts in DNA.

INTRODUCTION

The loss of enzyme-sensitive sites in DNA isolated at various times after irradiation has been used to measure the excision of thymine dimers in bacterial (1) and mammalian (2) cells. In this report we describe an analogous procedure for measuring the induction and repair of alkylation-induced enzyme-sensitive damage in DNA of Chinese hamster cells. We also measured the repair of alkylation-induced single strand breaks (or alkali-labile sites) in cellular DNA.

MATERIALS AND METHODS

Chinese hamster V79-379A cells in stirred suspension culture were labelled overnight with either [^{14}C]TdR or [^{3}H]TdR (3). ^{14}C-Labelled cells were treated with 0.12 mM dimethyl sulphate, dissolved in ether <0.1% and after various times aliquots were mixed with an equal number of ^{3}H-labelled cells that had been treated with solvent only. The DNA from the mixed culture was extracted from the pellet with a detergent solution and the protein removed by washing this extract with an equal volume of phenol solution (3). The aqueous layer was dialysed against a buffer and incubated with a crude extract of M. luteus [corresponding to fraction II of the preparation by Carrier and Setlow (4)] (or enzyme buffer for the controls) as previously described (3). Samples of the enzyme-treated and non-enzyme-treated DNA extracts (100 µl) were subjected to alkaline sucrose sedimentation and the number of breaks in DNA per 10^{8} daltons were calculated from

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the number average molecular weight (Mn) derived from T4 and T7 bacteriophage calibrated gradients (3).

RESULTS

1. Production and loss of single stranded breaks in alkylated DNA. ^{14}C-Labelled DNA from Chinese hamster cells 0.5 h after treatment with DMS (when the reaction was complete) was smaller than that of co-isolated ^{3}H-labelled DNA from untreated cells (Fig. 1). Post treatment incubation of alkylated cells during 24 hours resulted in an increase in size of alkylated DNA, up to that of DNA from untreated control cells. The difference between the number of breaks per 10^8 daltons in the DNA of alkylated and non-alkylated cells gave the number of alkylation-induced DNA strand breaks per 10^8 daltons. The number of these DNA single strand breaks decreased with time with a half-life of about 230 min during the first 6 h after treatment (Fig. 2).

2. Production and loss of enzyme-induced breaks in single stranded alkylated DNA. It can be seen in Fig. 1 that DNA as isolated from cells immediately after alkylation with DMS was further reduced in size following incubation *in vitro* with an extract of *M. luteus*. DNA isolated from untreated cells, when similarly incubated with the enzyme extract, was only slightly reduced in size. From the number of breaks present in these DNAs and in the non enzyme-treated, alkylated and non alkylated DNAs we calculated the number of breaks in DNA arising solely as a consequence of the action of the enzyme on alkylated DNA. The enzyme-sensitive sites in the alkylated DNA were lost with a half-life of about 130 min during the first 6 hours after treatment (Fig. 2). By 24 hours they had disappeared completely.

DISCUSSION

The extent of methylation of cellular DNA by a given concentration of DMS has not been measured. However DMS gives rise to the same methylation products in DNA as does methyl methanesulphonate (MMS) (5). It can therefore be presumed that DMS induces its cytotoxic effects in mammalian cells by the same mechanism as does MMS. DMS at a concentration of 0.12 mM, reduced the colony-forming ability of Chinese hamster cells to 20% of control (6). A concentration of MMS that reduced Chinese hamster cell survival to the same level methylated DNA to the extent of 1 μmole per gm of DNA (7). Thus in a piece of DNA of 10^8 daltons there are 100 methylation reactions, of which approximately 11% are recognized by the enzyme extract. DMS reacts with DNA to give a variety of

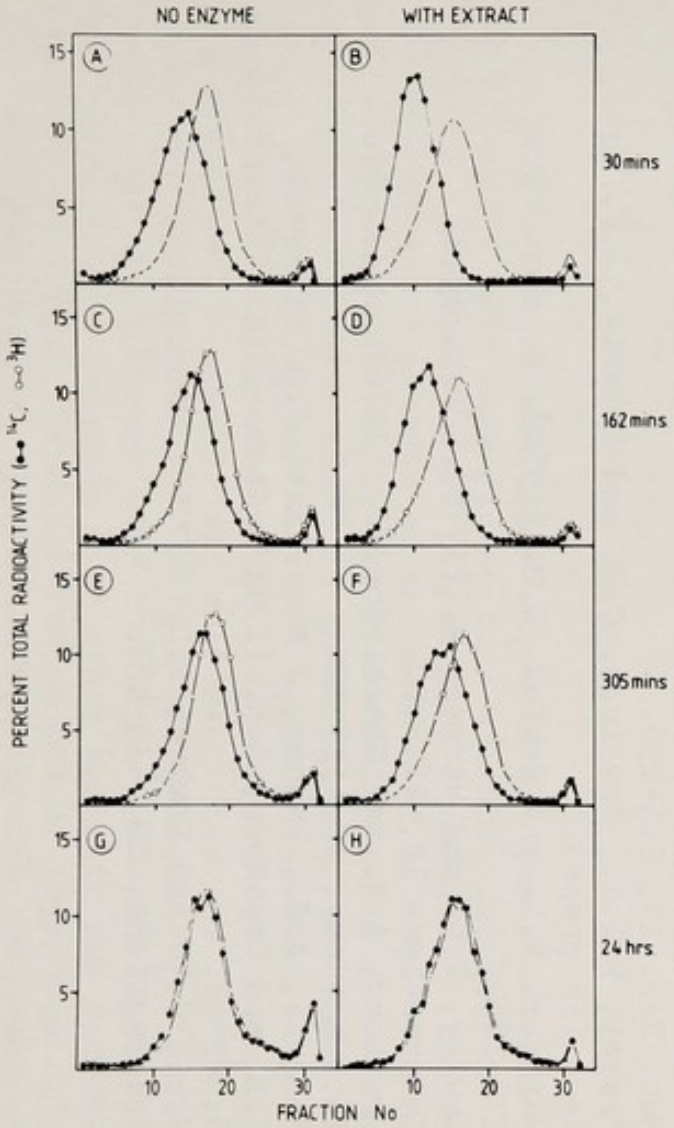

Figure 1.

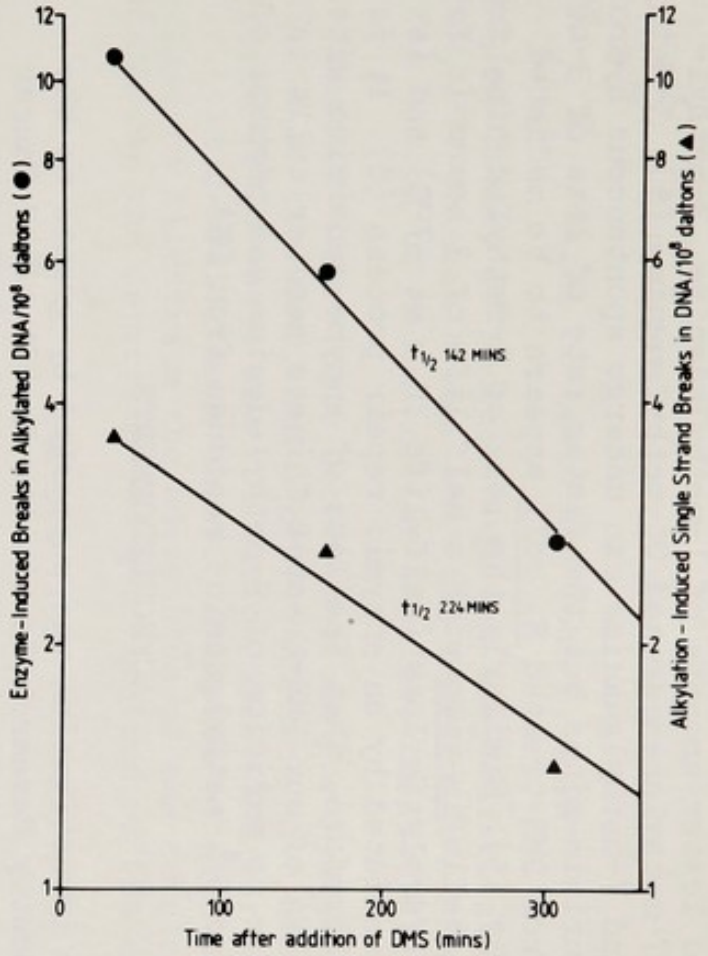

Figure 2.

products including 7-methylguanine (82%), 3-methyladenine (9.8%) and lesser amounts of 1-methyladenine, 3-methylguanine, 7-methyladenine and O^6-methylguanine (5). 3-Methyladenine and 7-methylguanine can undergo spontaneous hydrolysis to apurinic sites but the faster rate of loss of 3-methyladenine from DMS-treated E. coli appears to be mediated enzymically (5). Similarly the loss of 3-methyladenine from mammalian cells in vivo with a half life of 3 hours is faster than its hydrolytic loss (half-life 30 h at 37°C) and is probably mediated by an enzymic repair process (8). It is likely, therefore, that the loss of enzyme-sensitive sites from the DNA of our DMS-treated Chinese hamster cells is largely due to excision of 3-methyladenine and perhaps of 3-methyl- and O^6-methylguanine residues from DNA.

ACKNOWLEDGEMENTS

This investigation was supported by grants to the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital from the Medical Research Council and the Cancer Research Campaign.

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SEROLOGIC ASSAY OF DNA BASE DAMAGE[1]

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ABSTRACT Serologic determinations for hydroxymethyluracil, 8,5' cycloAMP, and O^6ethylguanosine have been developed using phage neutralization assay and for pyrimidine dimers, using radioimmune assay. Examples of each system are presented to illustrate the advantages of the serologic approach for the detection and quantitation of DNA damage.

INTRODUCTION

The application of serologic techniques to the study of unusual or altered bases began with the observation that antibodies to ruptured T4 phage were sufficiently specific for viral glucosylated methylcytosine to permit assay of phage synthesis in the presence of excess host DNA (1). A number of studies of minor bases and photoproducts has followed (2-5, as examples). Our efforts have chiefly been directed toward developing assays for ionizing radiation damaged bases (7).

RESULTS

Purines and pyrimidines serve as antigenic determinents, or haptenes, only when linked to a suitable immunogenic protein. Immunogens were prepared as a covalent linkage of the riboside and protein by the method of Erlanger and Beiser (8). Inocculation was by the method of Plescia (9). The phage neutralization assay is an adaption of Bonavida, et al. (10). Hydroxymethyluracil (HMU) has been identified as an irradiation product of thymine (11). The phage neutralization assay for HMU (Figure 1) was most sensitive for the deoxyriboside (HMUdR): 2×10^{-7} M (2×10^{-11} moles) were detected. Cross reaction occurred with thymidine and some analogues, but there was no reaction with other bases in DNA and RNA. Attempts to absorb the antiserum on DNA or thymidine columns has been ineffective. Increased sensitivity and selectivity have been obtained by absorption on Sepharose immobilized thymine riboside-protein conjugate in the presence of compet-

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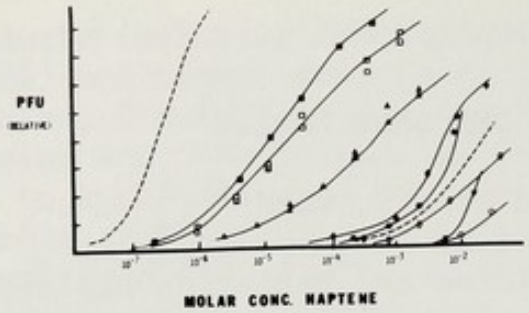

Figure 1

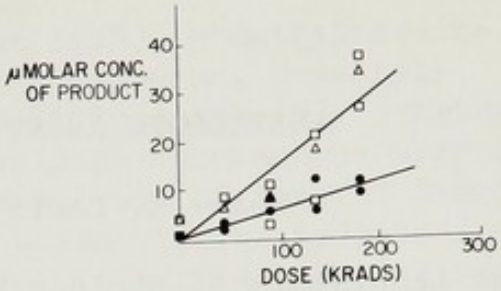

Figure 2

Figure 1. Phage neutralization haptene inhibition assay using anti-HMU serum. Inhibition by HMUdR, ■ : HMUR, □ ; HMU, ▲ ; TdR ● ; Thy ○ ; IUdR ◆ ; BrU ◇ ; and 6 methyl-uracil ▽ . The dashed lines are haptene inhibition curves using absorbed serum: HMUdR, far right; TdR, far left.

Figure 2. Phage neutralization assay of Co^{60} gamma irradiated thymidine (2 mgm/ml). Samples heated to 100°C at pH 9 prior to assay, □ ; samples treated with 10^{-3}M ferrous ions, △ ; heated at neutral pH ● . Catalase was added to all samples prior to assay to eliminate H_2O_2, which is toxic to phage.

ing free HMUdR. The results are the dashed lines in Figure 1. Interference by cross reacting species may be further reduced by saturation of the assay with excess thymidine.

Hydroperoxymethyluracil (HPMU) is a closely related species also produced by irradiation (12). Although it is unstable and not available in pure form for testing in haptene inhibition assay, it is possible to determine if it is part of the yield detected in irradiated thymidine. HPMU is converted to HMU by heating at high pH or by oxidation with ferrous ions. Figure 2 shows the effect of post-irradiation treatment on the yield. Conversion of the unstable product lead to a 3 fold increase in the yield, indicating that the antiserum did not react with the HPMUdR, but was specific for the HMUdR species. Since heating alone did not increase the yield, measurements made on heat denatured DNA will be of the HMU moiety alone. The G_{HMU} for native DNA irradiated in oxygenated solution was measured (after denaturation) as 0.05, essentially the same yield as irradiated TdR. We have confirmed this yield in irradiated DNA enzymatically hydrolysed prior to assay.

Raleigh, et al., have reported the formation of 8,5' cycloAMP in solutions of 5' AMP irradiated in the absence of O_2 (13). Dr. Raleigh has supplied us with 8,5' cycloadenosine for preparation of antiserum. The antiserum reacted poorly with 8,5' cycloAMP and assay of product formed by irradiation of AMP required prior enzymatic dephosphorylation. The yields of cycloAMP in AMP irradiated at varying pH in N_2 or N_2O are

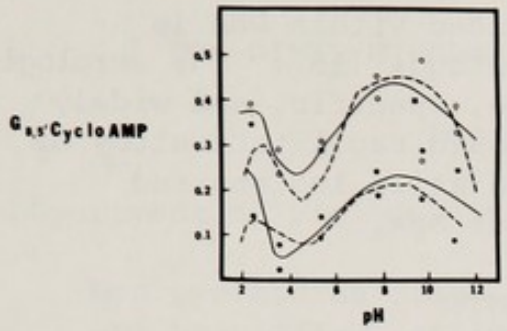

Figure 3

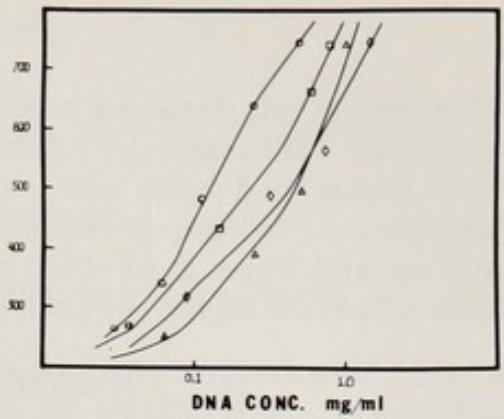

Figure 4

Figure 3. Effect of pH on the yield of 8,5' cycloAMP in solutions of AMP gamma irradiated in N_2, ● ; and N_2O, ○. The dashed lines are data of Raleigh, et al., (13).

Figure 4. Assay of DNA treated with 20 mM ENU ○ ; 10 mM ENU, □ ; 5 mM ENU, ◇ ; and 1 mM ENU, △ . The DNA was heat denatured prior to assay.

given in Figure 3 and are shown to agree with those of Raleigh (13). The chromatographic method employed by Raleigh for quantitation of product in the presence of excess parent compound was facilitated by the resistance of 8,5' cycloAMP to chemical hydrolysis. The serologic assay does not depend upon the chemistry of the bases and was applied to measurement of the cycloadenosine moiety produced by irradiation of related compounds. Although there is virtually no product formed in solutions of AMP irradiated in 0_2, there is a measureable amount formed by irradiation of AdR in 0_2 (G=.06). Yields in irradiated AdR and Adn in N_2 were: G=2.36 and G=.043, respectively. The cycloadenosine moiety has been detected in irradiated DNA in 0_2 and N_2.

0^6ethylguanine, a product of ethylnitrosourea (ENU) treatment of DNA, is of considerable interest since this lesion is not readily repaired in nervous tissue and results in specific tumor formation (14). Dr. Goth-Goldstein has prepared 0^6ethylguanosine for antibody production. The antiserum was highly specific showing no cross reaction with guanosine. N^7ethylguanosine or 1 methyladenosine. Figure 4 shows the haptene inhibition assay of DNA treated with 4 different concentrations of ENU. This preliminary test is promising as a sensitive assay which will not require expensive, radioactively labeled carcinogen.

L. McConlogue is using antibodies to UV irradiated DNA to study the distribution of UV lesion production and repair in mammalian cell chromatin. Using a radioimmune assay she has shown that the yield of measured products is lower in the staphylococcal nuclease resistant fraction of the chromatin.

DISCUSSION

The study of damaged bases produced within DNA is technically difficult by chemical methods (15). The serologic methods described here are sensitive, specific, and widely applicable. Further development should facilitate study of formation and repair of ionizing radiation lesions and mutagen altered bases within cellular DNA.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to Donn R. Muhleman for his excellent technical assistance and to Dr. Jim Raleigh and Dr. Regina Goth-Goldstein, for providing us with altered ribosides. We are indebted to Dr. Harvey Herschman for his council in immunological matters.

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NON-DIMER UV DAMAGE IN DNA AND POLY d(A-T)[1]

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Non pyrimidine dimer photodamage is introduced into DNA and poly d(A-T) at low doses of UV light. Irradiation of poly d(A-T) results in a form of damage unstable in the single-stranded conformation at neutral pH. Irradiation at 313nm in the presence of sensitizers suggests that the photodamage is derived from triplet state adenine.

INTRODUCTION

Although some attention has been given to non pyrimidine dimer DNA photoproducts (1,2), in general the high doses of UV necessary to introduce measurable levels of these products had seemed to preclude their being of biological significance. However, a number of recent studies on the biological effects of UV have suggested the presence of non dimer damage at physiological doses (3,4,5,6). The discovery in human placenta of a DNA binding protein specific for some non dimer damage (7) now provides a convenient and sensitive probe for the study of such damage.

METHODS

Filter binding assays and the purification of the placental UV-DNA specific binding protein were carried out as described by Feldberg and Grossman (7). Number average molecular weights were calculated from alkaline sucrose gradients by the method of Charlesby (8).

RESULTS

Dose Response of Binding. The dose-response for retention of T_7 DNA in the filter binding assay showed a typical saturation curve with total binding being obtained with a fluence of 50 J/m^2. A fluence of 12 J/m^2 gave 63% binding, suggesting, from the Poisson distribution, that damage was

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introduced at a rate of 0.21 sites per 10,000 base pairs per 10 J/m^2. With poly d(A-T) as substrate, a more gradual dose response curve was obtained with maximal binding (75% of total counts) occuring at a dose of 150 J/m^2. A dose of 54 J/m^2 was necessary to reach a level of 63% of maximal binding, suggesting (based on an Mw of 1 x 10^7 daltons) that damage was introduced into poly d(A-T) at a rate of 0.12 sites per 10,000 base pairs per 10 J/m^2.

Effect of Heat on Irradiated Polymers. Mild heating of irradiated T_7 DNA (75°C, 1 hour) had no effect on the ability of this polymer to bind to the placental binding protein. Heating irradiated poly d(A-T) to only 55°C, however, resulted in the time dependent loss of its substrate properties. Heating under alkaline conditions (pH 11.8, 55°C, 2 hours) followed by cooling and reneutralization had no effect on the substrate properties of irradiated poly d(A-T).

Analyzing the size of the heated poly d(A-T) by alkaline sucrose gradient centrifugation revealed a dose dependent decrease in molecular weight (Figure 1). From these data, the dose rate for introduction of breaks upon heating was calculated to be 0.06 breaks per 10,000 base pairs per 10 J/m^2. Centrifugation of irradiated heated poly d(A-T) on 70-100% formamide gradients also yielded a dose dependent decrease in sedimentation rate, suggesting that under neutral conditions chain breaks rather than alkali labile sites are introduced by heating.

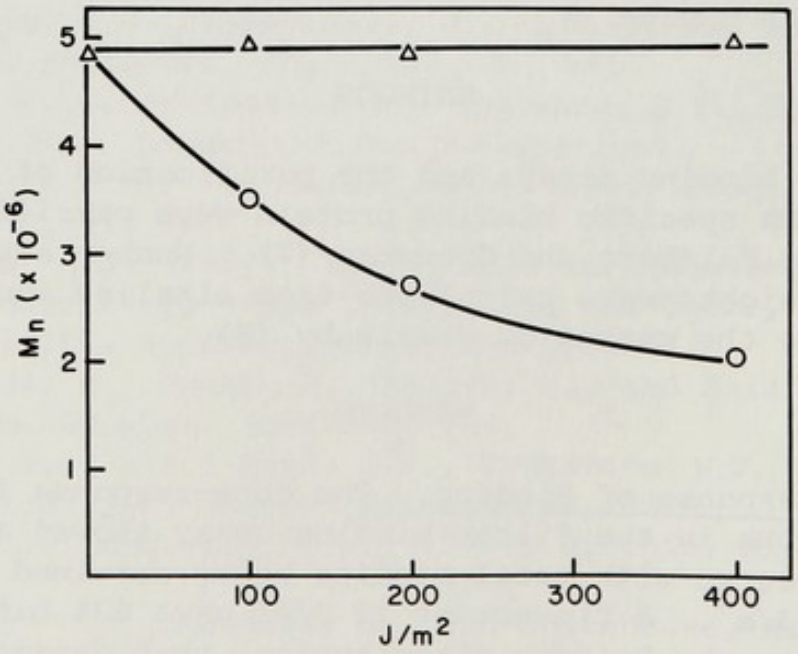

Figure 1. Effect of heating on irradiated poly d(A-T). Samples in 20 mM NaCl were either stored at 0°C (△) or were incubated at 55°C for 180 min (○). M_n analysis was carried out after centrifugation through 5-20% alkaline sucrose gradients for 180 min at 45k in the SW50.1 rotor.

TABLE I

Sensitized Irradiation of Poly d(A-T) and T_7 DNA[a]

DNA	Sensitizer	% cpm retained on filter
T_7 DNA	Acetophenone (.001M)	8
"	Acetone (1M)	36
"	---	10
Poly d (A-T)	Acetophenone (.001M)	8
"	Acetone (1M)	21
"	---	2

[a]Samples were bubbled with N_2 and irradiated in 2mm cuvettes for 20 min at 8.5 cm from a 100 watt medium pressure Hg arc lamp. All samples contained .05 mM Ag^+ and were assayed after dialysis using an excess of placental binding protein.

Sensitized Irradiation. Additional insight into the nature of the non dimer photodamage can be gained from experiments using 313 nm irradiation in the presence of triplet sensitizers. Damage recognized by the placental binding protein was found in T_7 DNA irradiated in the presence of acetone but not in the presence of acetophenone (Table 1). These data, together with the fact that damage is introduced into poly d(A-T) by 254 nm irradiation, suggested that the photodamage might indeed be derived from triplet state adenine. This conclusion is complicated by the poly d(A-T) results in which it is found that protein recognizable damage is introduced by both acetone and acetophenone.

DISCUSSION

Using a UV-DNA specific binding protein as a probe for non pyrimidine dimer damage, it has been possible to partially characterize such damage in T_7 DNA and poly d(A-T). Studies with T_7 DNA suggest a rate of damage introduction of 0.21 sites per 10,000 base pairs per 10 J/m^2. However, this value could be an underestimate if more than one damage site per T_7 molecule was necessary to ensure full retention of the DNA-protein complex on the filter.

UV-irradiation of poly d(A-T) also results in the introduction of protein-recognized damage. Heating the irradiated poly d(A-T) to its Tm value under neutral conditions resulted in the loss of protein binding as well as in the introduction of chain breaks. It should be pointed out that it is not yet clear whether the protein-recognized damage is the same in

poly d(A-T) and in DNA, nor whether the chain breaks in poly d(A-T) indeed originate from these photoproducts. However, the discrepancy between the T_7 and the poly d(A-T) sensitization data may be more apparent than real. The triplet energy level of acetophenone (26,500 cm^{-1}) is slightly above that of thymidine (26,300 cm^{-1}) and below that of adenosine (26,700 cm^{-1}). If excimer formation in poly d(A-T) (9) resulted in a slight stabilization of the adenine triplet state, it would indeed be possible for acetophenone to sensitize formation of triplet state adenine.

At this point, our working hypothesis is that we are dealing with an adenine photoproduct and that its lability in the single-stranded state suggests why this photoproduct has not been previously described.

ACKNOWLEDGMENTS

I would like to thank Mrs. Judy Lucas for her expert technical assistance during the course of this work.

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PHOTOCHEMISTRY AND PHOTOBIOLOGY OF DNA CONTAINING 5-IODOURACIL AND 5-IODOCYTOSINE[1]

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ABSTRACT The quantum yields for the photolysis of iodinated derivatives of cytosine (leading to the loss of iodine) has been measured for 313 nm radiation and found to decrease from 0.024 for IodCMP down to 0.007 for both Poly(C) and DNA containing 5-IoCyt. In DNA, approximately 1 clean chain break and 1 alkali-labile bond were formed for every 10 iodines lost. Incorporation of iodinated cytosine or uracil into the DNA of *Escherichia coli* or *Bacillus subtilis* was shown to have a large effect on the biological survival following 313 nm radiation.

INTRODUCTION

The substitution of 5-bromouracil (BrUra) for thymine (Thy) in DNA increases the sensitivity of cells to ultraviolet as well as ionizing radiation (1). This effect has been widely studied but the molecular lesion which is responsible for the increase in ultraviolet sensitivity is still in question (2). We have investigated the photochemistry of a similar system, namely DNA containing 5-iodocytosine (IoCyt), in order to help answer this question. In this paper we present the quantum yields for iodine photolysis and DNA strand breakage, using 313 nm radiation. Some preliminary data are also provided on the survival of *E. coli* and *B. subtilis* bacteria grown in the presence of 5-iododeoxyuridine (IodUrd) or 5-iododeoxycytidine (IodCyd) and irradiated in air at 313 nm.

METHODS

In vitro iodination of DNA with ^{125}I was done according to a method developed by Chan *et al.* (3) which minimizes the extent of chain scission. Thymidine requiring cells of *E. coli* B(3)T^- and either the wild-type or a mutant of *B. subtilis* (thy^-, trp^-) were grown in media containing IodCyd or IodUrd. Monochromatic radiation at 313 nm was obtained by passing the

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output of a 500-W mercury lamp through a Bausch and Lomb 0.5-m monochromator. The loss of iodine was measured either by following changes in the absorbance at 300 nm or by counting the ^{125}I activity left on filter paper discs following TCA precipitation and washing. Chromatograms were analyzed using a butanol:water:acetic acid solvent (80:30:12).

RESULTS

The absorbance changes at 300 nm accompanying the photolysis of IoCyt, IodCyd, IodCMP and iodinated poly(C) were used to calculate the quantum yields presented in Table 1. There

TABLE 1
QUANTUM YIELDS

Compound	Φ 313 nm
IoCyt	0.048
IodCyd	0.021
IodCMP	0.024
Iodo-Poly(C) (2%)	0.009
Denatured DNA	0.007
Renatured DNA	0.007-0.011
Iodo-Poly(C) (100%)	0.006

is approximately a 3-fold decrease in quantum yield upon photolysis of IodCyd in the polymer form. Spectroscopic as well as chromatographic evidence suggests that the loss of iodine from the C-5 position leaves a radical which combines with a hydrogen atom (abstracted from an as of yet unidentified source) to form cytosine. Oxygen when present competes favorably with this hydrogen abstraction mechanism for reaction at the 5-position, except in the polymer, resulting in oxidative degradation of the cytosine moiety. In the polymer, hydrogen abstraction is enhanced and the extent of oxidative degradation is diminished. However, the presence of oxygen had no effect on the quantum yield of photolysis in any case.

In poly(C) with 2% of its cytosine residues iodinated, the absorbance changes are too small to be measured and so the loss of iodine was measured by following the decrease in the ^{125}I activity associated with the polymer. The quantum yield as measured in this way agreed with that obtained spectroscopically. Loss of ^{125}I was also used to obtain the quantum yield for photolysis of various iodinated DNAs (both denatured and renatured) containing anywhere from 1 to 84% iodinated cytosine. As shown in Table 1, the quantum yield for photolysis of iodinated DNA is the same as for iodinated poly(C).

Chain breakage as measured by centrifugation in alkaline sucrose gradients accompanied loss of iodine from DNA. Approximately 1 break was formed for every 5 iodines lost. The frequency of breakage was reduced 2- to 4-fold by irradiating in the presence of either ethanol or cysteamine, which are good hydrogen atom donors. When the DNA was denatured with formaldehyde plus heat following photolysis and then spun on a neutral sucrose gradient, the frequency of chain breakage was reduced 2-fold. This result indicates that half the breaks observed on alkaline sucrose gradients represent alkali-labile bonds.

Iodinated DNA containing ^{14}C-Thy and ^{14}C-Cyt was extensively irradiated at 313 nm, degraded enzymatically to the nucleoside level, and the resulting hydrolysate analyzed by paper chromatography. The conversion of IodCyd to dCyd was observed as well as a comparable increase in the number of counts which remained at the origin. It is assumed that the material at the origin represents damaged regions of the DNA which are refractory to enzymatic digestion.

The influence of 313 nm radiation on *E. coli* and *B. subtilis* bacteria containing either IodUrd or IodCyd is shown in Table 2.

TABLE 2
BIOLOGICAL SURVIVAL

System	Irradiation	Supplement	% Survival
E. coli (T$^-$)	λex 313 (4,000 J/m^2)	2 μg/ml Thd	68
		+ 10 μg/ml IodUrd	0.4
		or 10 μg/ml IodCyd	1
E. coli (T$^-$)	λex 254 (16 J/m^2)	2 μg/ml Thd	37
		+ 10 μg/ml IodUrd	26
B. subtilis (T$^-$)	λex 313 (5,000 J/m^2)	10 μg Thd	90
		+ 100 μg/ml IodUrd	8
		or 100 μg/ml IodCyd	40
B. subtilis (wild)	λex 313 (15,000 J/m^2)	10 μg Thd	82
		+ 100 μg IodUrd	58
		or 100 μg/ml IodCyd	40
B. subtilis (Rec$^-$)	λex 313 (15,000 J/m^2)	0	22
		100 μg/ml IodCyd	No growth

Clearly the presence of these two halogenated compounds greatly sensitizes the bacteria to radiation at this wavelength. This sensitization effect is only slightly noticeable for 254 nm radiation. It is also obvious from Table 2 that incorporation of the IodUrd is necessary for sensitization since the wild-type strain of *B. subtilis*, which does not incorporate exogenous precursors readily, is not nearly as photosensitive as the thymidine requiring mutant.

DISCUSSION

The results presented here indicate that the quantum yield for photolysis of iodine in iodocytosine substituted DNA is nearly comparable with that of pyrimidine dimerization. The frequency of chain breakage in alkali relative to the loss of halogen is considerably less for iodocytosine containing DNA than for bromouracil substituted DNA for which the quantum yield for bromine disappearance equals the quantum yield of strand breakage (4). Clearly these differences reflect the more direct role played by the deoxyribose moiety in providing the hydrogen for abstraction at the C5 position for bromo- but not for iodo-photolysis.

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DNA REPAIR PATHWAYS: EXCISION REPAIR IN PERMEABILIZED CELLS[1]

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ABSTRACT Schemes proposed and documented for the repair of structural defects in DNA are outlined. It is important to consider unique effects in replicating regions of DNA. Lesions on the leading strand side may be treated differently than those on the lagging strand side of the fork. Excision repair involves the removal of damaged single strand regions within duplex DNA and resynthesis. Studies with permeabilized bacteria have aided the analysis of precursor, energy and enzyme requirements for excision repair. For example, previous results with toluenized *E. coli* have shown that either pol II or pol III can substitute for pol I in an ATP dependent mode of repair replication. We have developed an analogous approach for studying excision repair in permeabilized human cells. Isolated WI38 nuclei exhibit UV dependent repair replication while nuclei from xeroderma pigmentosum (group A) cells do not. Added T4 endonuclease V, but not pancreatic DNase I, promotes UV dependent repair synthesis. Our results support the view that the incision step in the excision repair sequence is defective in xeroderma pigmentosum (group A) and they demonstrate the utility of permeabilized mammalian cells for probing DNA repair with exogenous enzymes and substrates.

INTRODUCTION

The variety of pathways that have been proposed for negotiating structural defects, such as pyrimidine dimers, in the DNA of viable systems can be reduced to a small number of essential approaches (1). We should distinguish between those schemes that restore the DNA to its undamaged functional state and those that promote recovery without intentionally removing

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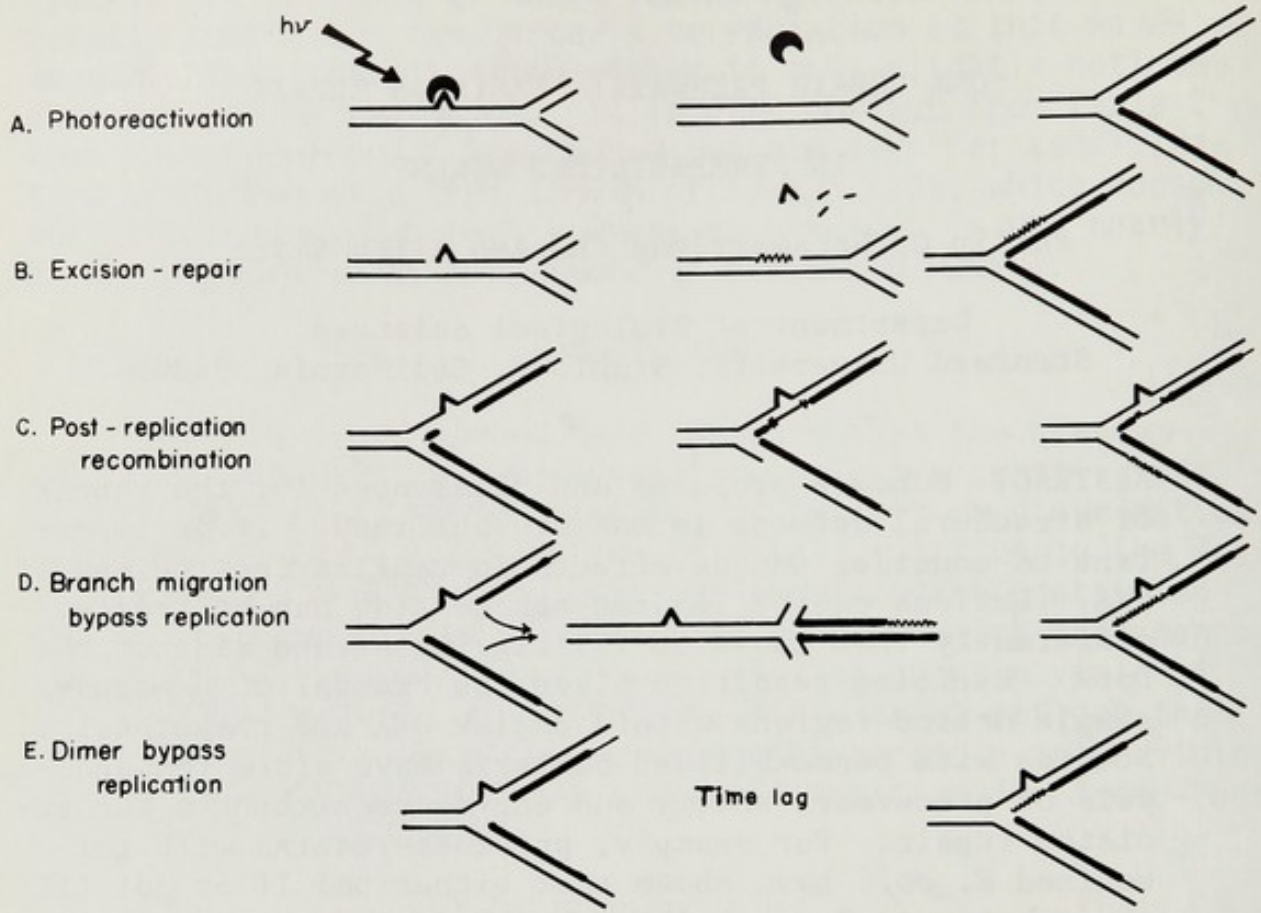

FIGURE 1. *Schemes for repairing or bypassing dimers.* Repair replication is shown by wavy lines as distinguished from normal replication shown by heavy lines.

the damage. We should also consider the relative importance of different schemes in terms of their enhancement of survival. Using the "classical" pyrimidine dimer as test lesion (Fig 1) we outline the possibilities. The dimer may be reversed *in situ* by photoreactivation (A) or it may be deleted by excision (B), both of which processes normally require enzymatic recognition of the defect. Photoreactivation is error-free, unless further chemical alteration (eg. deamination of dimerized cytosine) has occured prior to reversal of the dimer. Excision repair should be as error-free as the polymerase that promotes the resynthesis step since the complementary parental template strand is usually intact. We could also conceive of a fortuitous excision repair mode in which the dimer is not specifically recognized but in which local structural distortion of the DNA results in nicking by a single strand-specific endonuclease in the vicinity of the dimer, with the same ultimate result. The bypass schemes, C,D and E in Fig. 1 do not really constitute repair at all since the dimer remains in the DNA.

Although they do result in the gradual dilution of the damage and the eventual generation of dimer-free daughter DNA they do not necessarily represent significant recovery modes in terms of survival. They all involve sequences of events that begin in the growing fork regions of DNA. However, recombination (C) could operate in any situation in which homologous DNA sequences are present. Unique features of replicating regions (eg. gaps in daughter strands) may promote that process. Branch migration bypass replication (D) has been postulated (2) and although not yet established as a recovery process, it is of interest as a possible error-free mode of replication past dimers. Any attempted direct bypass replication (E) should be error-prone *a priori* since a dimer does not exhibit the structural features of its constituent pyrimidines. The SOS model proposed by Radman involves an inducible suppression of the editing 3'-5' exonuclease of DNA polymerase so that the enzyme is less stringent in its requirement for appropriate base pairing (3) (Radman et al, this volume). Still another inducible response, specific for alkylation damage and distinct from SOS repair, has been recently described (4).

EFFECTS IN REPLICATING REGIONS

Since an *E. coli* cell may be killed by as few as one or two unrepaired dimers in its chromosome the precise location of a dimer may be significant. A dimer poses a block to replication in the sense that DNA synthesis overall is slowed in UV irradiated cells. However, we know very little of the detail of this inhibitory effect at the molecular level. The observation of gaps in daughter DNA strands synthesized on dimer-containing templates indicates that the inhibition must not be complete. In some systems such as UV irradiated simian virus 40 gaps are not observed but replication eventually overcomes the dimer after a long lag (Sarasin and Hanawalt, this volume.) What actually happens may depend upon whether the lesion is on the leading strand side or the lagging strand side of the growing fork. Figure 2 illustrates the important difference as first pointed out by Meneghini (5). A block on the leading strand side should have no effect on replication in the lagging strand and *vice versa*. However, if replication is continuous on the leading strand, a dimer must result in a very extensive region of unpaired parental DNA. In contrast a dimer on the lagging side should leave a gap no larger and generally smaller than an Okazaki fragment. It remains to be determined if and when the parental strand unwinding rate is affected by either or both of these situations. In excision proficient systems the generation of DNA with daughter-strand gaps opposite dimers in parental strands would seemingly pose an additional hazard. However, it is possible that the dimer-

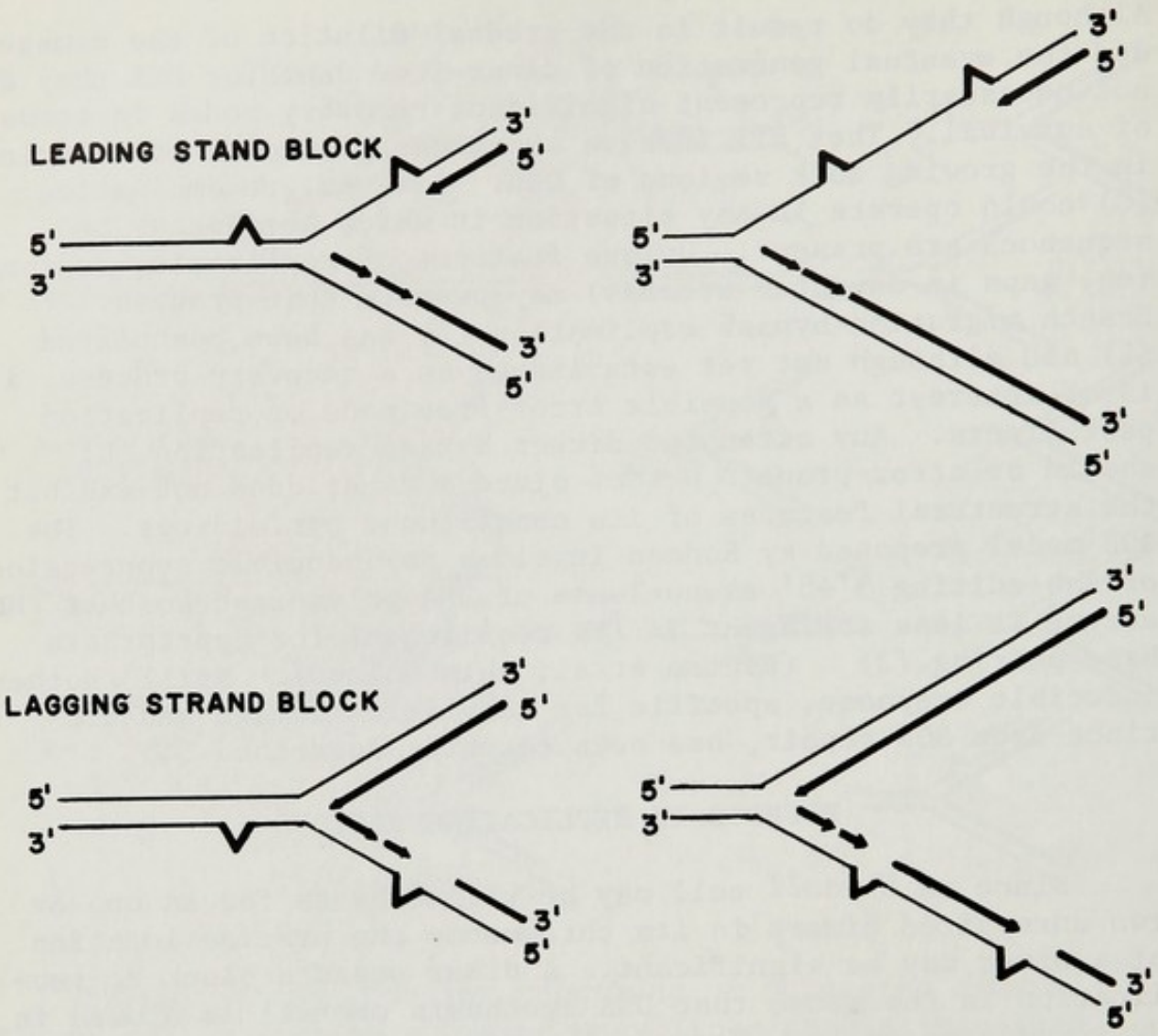

FIGURE 2. *Effect of a dimer on DNA replication.*

specific endonucleases can not operate on dimers in the configuration represented in Fig. 2. (ie. adjacent to the end of a growing chain). Thus, dimers thought to be in this configuration in WI38 human fibroblasts were found insensitive to the T4 endonuclease V (6).

EXCISION REPAIR IN E. COLI

The coordinated steps of excision repair in *E. coli* are apparently carried out most efficiently by DNA polymerase I and its associated 5'-3' exonuclease, following the initial endonuclease scission. An early indication of the operation of alternative enzymes was the observation of enhanced "long-patch" repair replication in *pol* A mutants (7). We now know that other enzymes can substitute for both the polymerizing and the excising roles of pol I, with the usual result that a longer average patch size ensues (8). The complexity of the

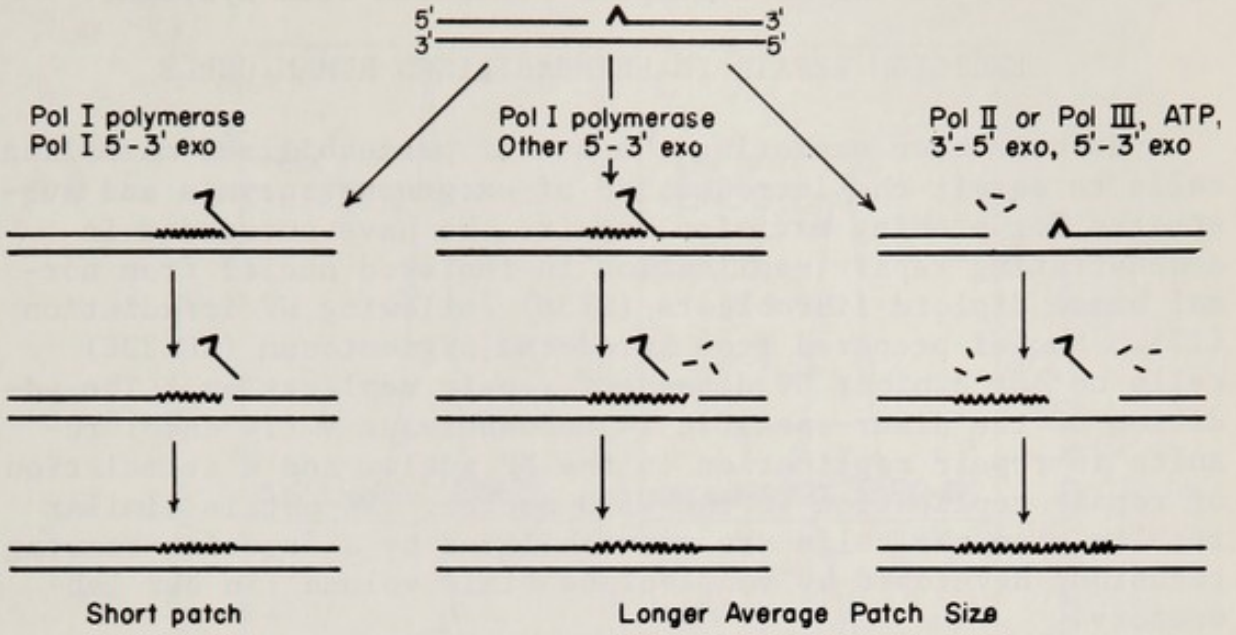

FIGURE 3. *Pathways for excision-repair in E. coli.*

alternative pathways is illustrated in Figure 3, which indicates the need for a gapped DNA substrate for the operation of pol II or pol III. Not shown is the possibility suggested by Friedberg (personal communication) that excision might be promoted by a single-strand specific endonuclease, operating on the dimer-containing "tail" of parental DNA. However, *in vitro* studies (9) and *in vivo* studies (Cooper and Hunt, this volume) indicate that dimer excision is most efficiently carried out by the coupled activities of polymerase and exonuclease. Interestingly, the polymerizing activity of pol I seems to be more essential than its own 5'-3' exonuclease activity for dimer excision. (Cooper and Hunt, this volume). Possibly the functional polymerase component can "drive" several alternative exonucleases such as exonuclease V and exonuclease VII. (Masker and Chase, this volume). The pol I independent excision repair pathways have been shown to require protein synthesis and *recA* $^{+}$ (10). Studies reported by Cooper and Hunt (this volume) and Ben-Ishai et al (this volume) suggest that the pol I independent long-patch component of excision repair may be one of the inducible SOS functions (3). Our earlier studies with toluene permeabilized *E. coli* had shown that either pol II or pol III may carry out repair replication in pol I deficient strains and that ATP or another nucleoside triphosphate cofactor is essential (11). The studies on excision-repair in *E. coli* have benefited from the existence and construction of a large catalogue of mutant strains. The bacterial results continue to provide useful guidelines for anal-

ogous studies on the more complex mammalian cell systems.

EXCISION REPAIR IN PERMEABILIZED HUMAN CELLS

We have been exploring the use of permeabilized mammalian cells to permit the introduction of exogenous enzymes and substrates for probing excision repair. We have succeeded in demonstrating repair replication in isolated nuclei from normal human diploid fibroblasts (WI38) following UV irradiation (12). Nuclei prepared from xeroderma pigmentosum (XP12BE) cells do not exhibit UV dependent repair replication. The addition of the dimer-specific T4 endonuclease V (T4 endo) results in repair replication in the XP nuclei and a stimulation of repair replication in the WI38 nuclei. We obtain similar results when the cells are permeabilized by a freezing-thawing technique developed by van Zeeland (this volume) in our laboratory.

Our detailed protocol for DNA repair experiments with isolated nuclei is described elsewhere (12). The cells are prelabelled with ^{32}P and incubated in 10 μM BrdUrd and 1 μM FdUrd for several hours prior to UV irradiation and preparation of nuclei. Nuclei are prepared by rinsing three times with 5 ml and then incubating the cells for 10 min at 4° in 3 ml buffer containing 5 mM $MgCl_2$, 1 mM EDTA, 6mM 2-mercaptoethanol, 10 mM Tris, (pH 8 at 20°). Two ml is removed and held in a Bellco "shortie" pipette. The monolayer is scraped down and pipetted against the plate 4 to 5 times in the reserved buffer, and held at 0°. Nuclei are harvested from combined similarly treated plates by centrifugation at 2500 rpm for 3 min at 20° in an International UV centrifuge and then resuspended in fresh buffer by brief vortexing. The resuspended nuclei are assayed in a mix containing in 0.5 ml, 7 mM Tris, 10 mM $MgCl_2$, 0.1 M NaCL, 4 mM 2-mercaptoethanol, 0.7 mM EDTA, 5 mM ATP, 0.1 mM each dATP, dCTP, dGTP, 10 μM BrdUTP, 30 mM HEPES and 2 μM[^3H]-dTTP at 100 μCi/ml. The pH is 7.8 at 37°. Samples for assay are centrifuged, resuspended in 2.5 ml of 10 mM Tris, 10 mM EDTA, 0.5% sodium dodecyl sulfate, 50 μg/ml Proteinase K and incubated 2 hr at 50°. The digests are sheared by 2 passes through a 25 gauge needle and the DNA is resolved into parental and normally replicated (hybrid) bands by equilibrium sedimentation in neutral CsCl gradients. The $^3H/^{32}P$ ratio in peak fractions of parental density DNA from alkaline CsCl rebandings is taken as a measure of repair replication (13).

An example of our results with XP12BE nuclei prepared from contact-inhibited cells is given in Figure 4. These repair-deficient cells perform essentially no repair synthesis in our whole cell assay. It is evident that significant repair synthesis occurs only when the T4 endo is added to the

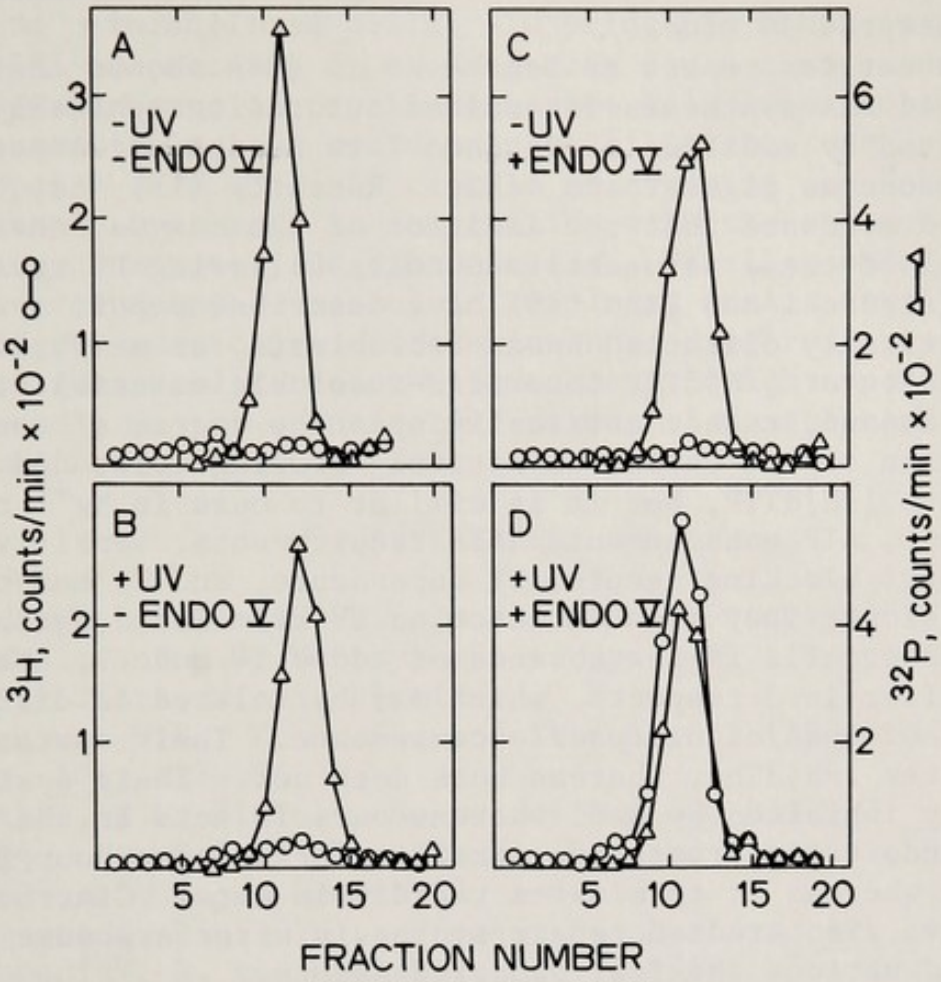

FIGURE 4. *Repair replication in XP12BE nuclei.* Alkaline CsCl gradient profiles of purified parental density DNA. 30 J/m^2 UV was 30 min prior to preparation of nuclei.

permeabilized system, supporting the view that the initial endonuclease step is deficient in these cells. Nuclei from UV irradiated WI38 cells exhibited repair synthesis for a limited period without added T4 endo, but synthesis was stimulated and extended when the enzyme was added. In control experiments it was shown that added pancreatic DNase I which produces a similar type of scission did not stimulate UV dependent repair synthesis significantly. The T4 endo stimulated synthesis persists for an hour, requires Mg^{++}, and is ATP dependent. The synthesis utilizes [^{3}H] dTTP but not [^{3}H] dThd. The use of BrdUTP and density analysis of the DNA in our studies clearly identifies the observed synthesis as of the repair mode. In fact it has been possible to estimate the average patch size by rebanding the parental density DNA

in alkaline CsCl after shearing by sonication (12). We find the repair label in stretches of roughly 35 nucleotides, the size measured *in vivo*.

An earlier report of Tanaka *et al* (14) showed that unscheduled DNA synthesis determined autoradiographically was stimulated by addition of T4 endo V to Sendai virus permeabilized xeroderma pigmentosum cells. Recently (15) they have provided evidence that the addition of the enzyme enhanced the survival of these permeabilized cells following UV irradiation.

Ciarrocchi and Linn (16) have described repair synthesis in osmotically disrupted human fibroblasts, as measured by incorporation of [^{3}H]dTTP into acid-insoluble material. Their system depends rather critically upon the degree of contact inhibition of the cells, as actively growing cells did not incorporate [^{3}H]dTTP, but it is similar to ours in Mg^{++} requirement, ATP enhancement, dNTP requirements, sensitivity to sulphydryl blocking agents, UV dependence, and T4 endonuclease stimulation. They also detected no UV stimulated synthesis in XP group A cells in the absence of added T4 endo V. The systems differ in 3 respects, which may be related to differing degrees of loss of diffusable components. Their system incorporates [^{3}H]dThd, whereas ours does not. Their system is strongly inhibited by NaCl whereas ours is not. In the absence of T4 endo V, incorporation persists for over an hour in their system, whereas it terminates rapidly in ours. Ciarrocchi and Linn have also studied repair synthesis after exposure to X-rays and various chemical damaging agents.

Trypsinized cells may also be rendered permeable by treatment with Tween 80 or by rapid freezing and thawing in the hypotonic buffer used for nuclei preparation, immediately prior to addition of substrate mix. Our results with freezing and thawing generally parallel those with nuclei, except that repair replication is greatly diminished in cells irradiated immediately prior to freezing, consistent with the notion that the human endonuclease is inactivated by freezing. Incorporation in this system appears to be linear for a longer period of time than with nuclei, possibly reflecting a slower rate of diffusion out of necessary cellular constituents. Repair replication has been observed in cells treated with various DNA damaging chemicals prior to freezing and thawing (A. Lehmann, pers. comm).

The density labelling method allows clear resolution of repair synthesis from semiconservative synthesis, may be utilized with growing cells, and allows further detailed characterization of the synthesis performed. For rapid screening of cell lines and other treatment conditions determination of [^{3}H]dTTP incorporation into acid-insoluble material or modification of the density labelling method to include only a single alkaline CsCl gradient may be more appropriate.

The examples given illustrate the utility of probing DNA repair defects by the addition of exogenous enzymes and substrates to permeabilized cells. In principle other enzymes such as DNA polymerases or exonucleases could also be added to compare performance of repair-defective cell lines to that of normal human cells. It would even be of interest to determine whether pol I of *E. coli* is capable of carrying out "short patch" repair synthesis in competition with the endogenous mammalian polymerases and exonucleases. Finally, this quasi *in vitro* system may be useful in resolving qualitatively the nature of the respective defects in those different complementation groups of xeroderma pigmentosum, which appear to differ merely in the quantitative extent of damage removal and repair synthesis.

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A NEW CLASSIFICATION OF PATHWAYS REPAIRING PYRIMIDINE DIMER DAMAGE IN DNA[1]

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ABSTRACT Pathways of repair of pyrimidine-dimer damage are classified as intrareplicational if they operate on incompletely replicated segments of a chromosome and extrareplicational if they operate on unreplicated and completely replicated segments. Intrareplicational repair is divided into four pathways: 1. Transdimer synthesis (formerly SOS repair, mutation-prone repair and induced repair) 2. & 3. RecBC and alternate RecF pathways of breakage-reunion gap-filling (formerly postreplication repair and recombination repair) 4. Copy-choice excision (a new proposal). Extrareplicational repair is divided into three pathways: 1. Short patch excision which operates on unreplicated and completely replicated chromosome segments 2. Long patch excision which may operate only on completely replicated segments and 3. Incision-promoted-recombinational excision which may be the mechanism of long patch excision. Reliance of these pathways on particular genes is discussed as is their possible evolutionary significance.

INTRODUCTION

Currently there are in use several schemes to classify pathways which repair pyrimidine dimer damage in DNA (2,18,49). Significant genes such as uvrA and recA have been used to separate pathways into those dependent upon one or the other. Key differences in the results of repair such as excision of damage, recombination, and mutation have been used as criteria for classification. Regulatory differences (inducible vs. constitutive) form the basis of another classification scheme.

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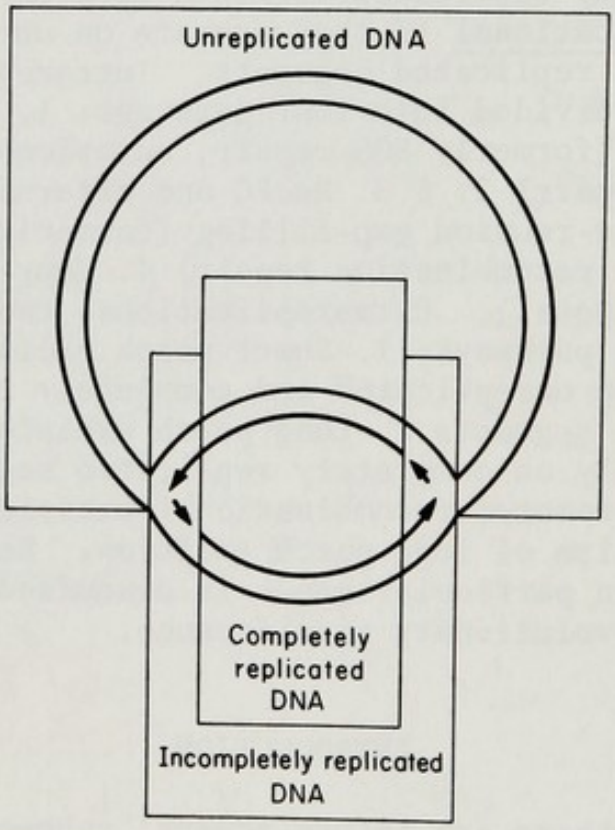
In addition operational criteria such as incubation in the dark or light, teleological criteria such as SOS or tolerance and interpretive criteria such as postreplication have been used to classify pathways. All of these are valid and useful in promoting an understanding of DNA repair pathways. We have, however, found them limiting in that pathways whose existence would explain or be consistent with experimental evidence seem to lie outside of their borders. As a consequence we wish to propose another classification of DNA repair pathways because it seems all inclusive and its exploration has revealed to us conceivable pathways whose existence would assist in accounting for experimental data.



Intrareplication Repair ≡ Repair of incompletely replicated DNA

Extrareplication Repair ≡ Repair of completely replicated and unreplicated DNA

FIGURE 1. Classification of DNA repair pathways. The heavy lines represent the strands of a chromosome which is replicating bidirectionally. Three regions are distinguished by their relationship to the replication fork and their replicative state. Arrowheads denote 3'termini.

In this classification scheme, shown in Fig 1, DNA repair pathways are classified on the basis of the chromosomal regions on which they operate. Each replicating chromosome can be divided into unreplicated, incompletely replicated and completely replicated regions. Unreplicated and completely replicated regions differ in that two complete copies of the latter and only one copy of the former are part of the chromosome. Incompletely replicated regions are those in which at least one copy is not complete. Pathways of repair operating on incompletely replicated regions we call intrareplicational because they are within the boundaries of the replication process. Pathways of repair operating on unreplicated and completely replicated regions we call extrareplicational because they are outside the replication process. Another way of looking at this classification is that unreplicated damaged DNA will be incompletely replicated unless the damage is removed or its inhibitory effects otherwise mitigated by extrareplication repair. The replication of the incompletely replicated damaged DNA will be completed by intrareplication repair. Finally completely replicated damaged DNA is subject to extrareplicational repair.

We have chosen the designation "intrareplication" in place of the traditional "post-replication" because the function of the repair is to complete the replication of incompletely replicated segments of chromosome. Throughout this review we have named pathways by a phrase descriptive of specific intermediates, mechanisms, or end products. We have avoided commonly used names such as inducible or error-prone because they refer to several pathways and hence tend to be equivocal.

In reviewing the pathways of intrareplication and extrareplication repair of UV damage we have chosen to make four simplifying omissions: (1) we deal only with E. coli, (2) we consider only one type of damage introduced by UV irradiation, i.e. pyrimidine dimers, (3) we omit pathways which function on segments containing more than one dimer, and (4) we omit pathways which require light to function, e.g. enzymatic photoreactivation.

INTRAREPLICATION REPAIR

uvrA or uvrB mutants are known to be incapable of removing pyrimidine dimers from DNA (2,21,41); Rupp and Howard-Flanders (37) added radioactive DNA precursor to a uvrA mutant following exposure of the mutant to UV irradiation. The label was incorporated into short fragments of DNA, which were detected in alkaline sucrose gradients, because replication on damaged templates was periodically interrupted (Fig 2). Continued incubation in the absence of labeled

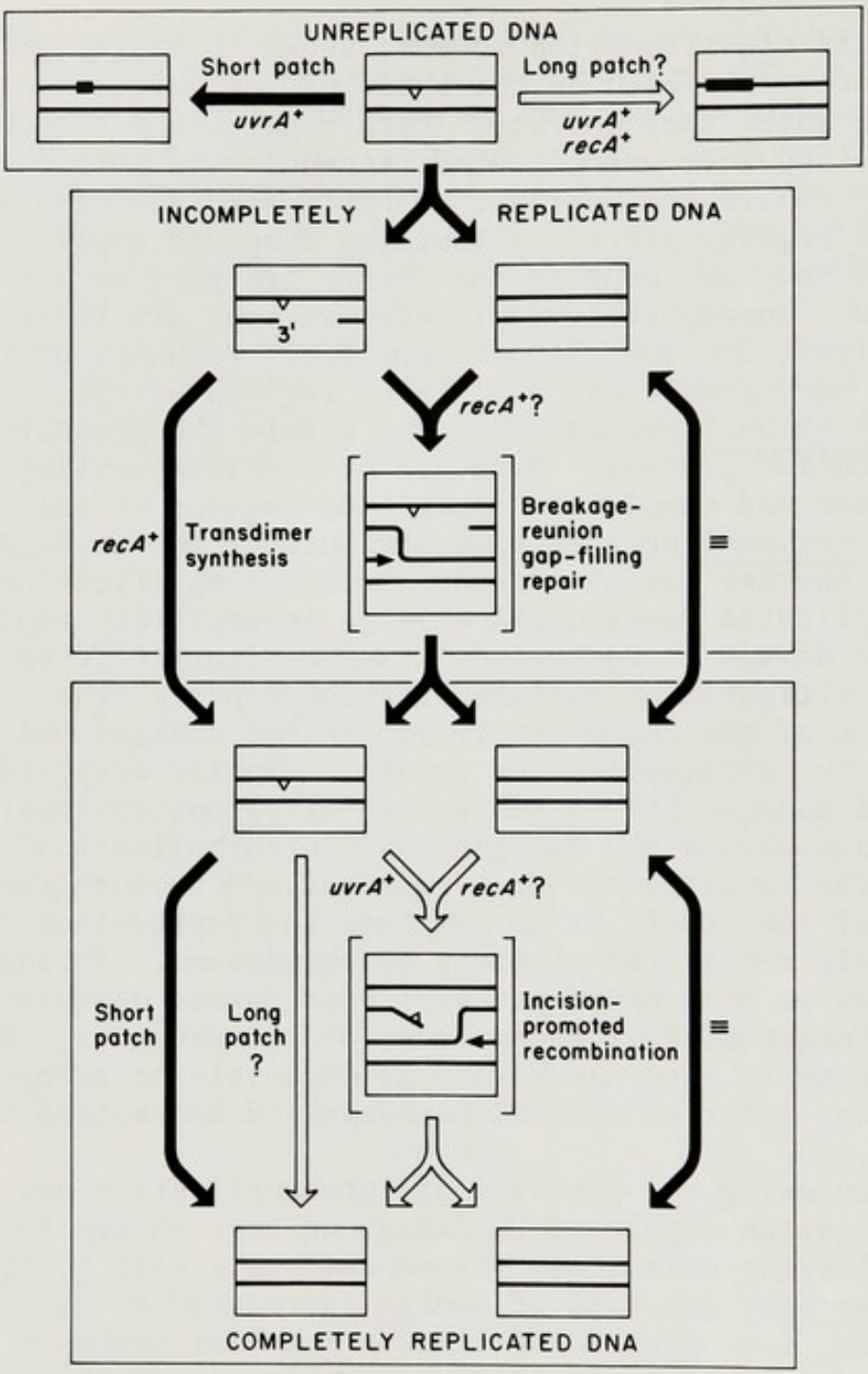


FIGURE 2. Hypothetical pathways of intrareplicational and extrareplicational repair. Chromosome regions (Fig. 1) are circumscribed by large boxes. Small boxes limit the view to monodimer segments within those regions. The following symbols are used: (——), DNA strands; (—■—), amount of DNA replaced during dimer excision; (—▽—), a pyrimidine dimer; and (——►), a 3'terminus being extended by polymerization; (■►), pathways about whose location we are certain; (⇨), pathways about whose location we are uncertain; and (≡), identity of structures. The number of steps in the pathways are not indicated. Hypothetical intermediates are in brackets.

precursor led to an increase in size of the labeled fragments as the discontinuities were repaired and replication completed. Later Rupp et al (38), showed that recombinational exchange between daughter strands and parental strands was correlated with the increase in size of the labeled daughter strands. Such recombinational events were conceived to involve interaction of both copies of incompletely replicated chromosome segments and at least two strand breakage and joining events (Fig 2). We call this type of pathway breakage-reunion gap-filling repair.

An alternative route to accomplish gap filling is suggested by studies of repair of the UV-irradiated single-stranded phage ϕX174. This repair pathway is thought to be due to the induction of one or more proteins capable of inhibiting the 3' to 5' correction exonuclease activity of one or more of the E. coli DNA polymerases (P. Caillet-Fauquet, M. Defais and M. Radman, Personal communication). The blockage of the proofreading function of the correction exonucleases will prevent the removal of those incorporated bases which are not hydrogen-bonded to the complementary strand in a normal configuration. Thus the polymerases would presumably be able to complete synthesis on a damaged template. We call this type of pathway transdimer synthesis.

At present the amount of intrareplicational repair which is attributable to breakage-reunion gap-filling and to transdimer synthesis can not be specified with certainty. It appears that recA mutations block both induced reactivation of ϕX174 (11) and the increase in size of DNA labeled after UV irradiation (42). For this reason we have included recA$^+$ in both pathways (Fig 2). We are uncertain whether recA controls an early or a late step in breakage-reunion gap-filling (24).

ERROR-PRONE REPAIR

The presence of induced editing inhibitors in UV-irradiated cells would render error-prone all DNA synthetic pathways using susceptible polymerases. This applies to replication of undamaged DNA as well as to the repair of damaged DNA (22,47,48; Radman, personal communication). Given this possibility we have wanted to avoid the confusion attendant upon naming any one pathway the error-prone or the mutagenic repair pathway.

PATHWAYS OF EXTRAREPLICATION REPAIR

Intrareplication repair pathways cope with the effects of pyrimidine dimers on replication. By contrast extrareplication

repair pathways serve to remove dimers from unreplicated and completely replicated chromosome segments. Damaged chromosome segments which have undergone intrareplication repair retain their pyrimidine dimers and are thus converted from unreplicated damaged DNA to completely replicated damaged DNA. Although both are substrates for extrareplication repair pathways, completely replicated but not unreplicated damaged segments may utilize recombinational repair pathways because of the possible homologous interactions between the two complete copies per chromosome of the completely replicated segments.

Possible pathways of extrareplication repair are also shown in Fig 2. All are considered to involve excision of pyrimidine dimers and to depend on uvrA$^+$ and uvrB$^+$. Cooper and Hanawalt (9) have divided excision repair into two categories, short patch and long patch, based on the size of the excision gap. Since the intermediates proposed (8,18) do not involve recombinational interactions these pathways might be expected to repair both unreplicated and completely replicated DNA. A recombinational pathway of excision repair operating on completely replicated DNA is also conceivable. The nicked strand containing a pyrimidine dimer is used as the source of DNA for synapsis with the undamaged sister replica. A combination of strand displacement and strand assimilation (32) would eliminate the dimer. New synthesis would occur on the originally undamaged dupl

The recombinogenic qualities of a nicked dimer-containing molecule have been pointed out by Ross and Howard-Flanders (33,34) on the basis of recombination-initiating qualities of molecules containing psoralen cross-links. The same proposal is implicit in the findings of Helling (19) who showed a uvrA$^+$ uvrB$^+$ recA$^+$ dependent stimulation of transduction frequencies when a P1 lysate was UV irradiated. Presumably donor DNAs which would have been inherited unilinearly in abortively transduced clones were nicked at the sites of dimer damage and used to produce complete transductants. We call the pathway suggested by these results incision-promoted recombinational excision repair.

Short-patch excision repair operates in a recA13 recB21 strain and is therefore recA independent (10). Long patch excision, however, is dependent upon recA$^+$ since it does not occur in a recA13 recB21 (10) but does in a recA$^+$ recB21 strain (P. Cooper, personal communication). If long patch repair operates only on completely replicated chromosome segments, it may be incision-promoted recombination; if it operates on unreplicated segments there may be two types of long patch repair.

CONTROL OF REPAIR PATHWAYS

Three types of experiments reveal that there are both constitutive and either inducible or activable pathways. The first type is the use of chloramphenicol to inhibit protein synthesis following UV-irradiation. Sedgwick (40) has shown that some intrareplication repair is inhibitable by chloramphenicol. P. Cooper (personal communication) has shown that some long patch synthesis accompanying excision is likewise inhibitable. One precaution is that cells to be tested must be incubated in the presence of chloramphenicol for at least 15 min prior to irradiation in order to attain the maximal effect (12; M. Radman, personal communication). P. Cooper (personal communication) has found increased inhibition of long patch synthesis by such preincubation. Sedgwick added chloramphenicol "immediately before" irradiation and might expect increased inhibition of intrareplication repair upon earlier addition.

The second type of experiment is to infer the existence of inducible repair from the greater reactivation of UV-damaged phage which infect UV-irradiated rather than unirradiated host cells (46). This UV-induced or W (for Weigle) reactivation is seen both for double-stranded DNA phage (e.g. Lambda) (46) and for single-stranded DNA phage (e.g. S13) (43). The reactivation of the lambda has been found to be inhibited by chloramphenicol added 15 min before irradiation of the host cells (12).

The third type of experiment is to determine the genes on which a given pathway depends and infer from the known or hypothesized action of these genes the type of control exerted on the pathway. recA and lexA genes are particularly pertinent because of the hypothesized regulatory interaction of their products, (15,16,28). According to the theory, lexA determines a repressor of a set of genes which includes recA. The RecA protein is activated following UV irradiation and proteolytically cleaves a set of repressors (including LexA) thereby derepressing not only its own synthesis but the synthesis of all other products controlled by lexA and all other products controlled by the other susceptible repressors.

Since it is part of the theory that activated RecA protein inactivates repressor proteins by proteolytic cleavage, it is tempting to consider all pathways dependent on $recA^+$ and $lexA^+$ to be UV-inducible (i.e. UV-derepressible). This would also appear to be an appropriate classification of those pathways inhibited by chloramphenicol. It has not yet been ruled out, however, that activated RecA protein also activates constitutive proteins proteolytically. It may even be the

case that a cleaved repressor (e.g. the LexA product) is activated to perform another function. In the case of chloramphenicol inhibition it may be that derepressed levels of RecA protein are needed to activate the constitutive repair proteins and it is the derepression of recA which is inhibited. As a result it seems advisable to classify recA and lexA dependent pathways and chloramphenicol-inhibitable pathways as UV inducible or UV activable. It will be necessary to reexamine this classification should it be found that RecA and LexA proteins participate directly (either catalytically or stoichiometrically) in repair.

ROLE OF recF IN REPAIR

Mutations in the recF gene (20) have been found to confer a phenotype similar to that conferred by lexA mutations. Among other similarities both cause UV sensitivity as single mutations (20,29). As determined by formation of conjugational recombinants, both block recombination in a recB21 recC22 sbcB15 genetic background and fail to in a $recB^+$ $recC^+$ $sbcB^+$ background (20,29, A.J. Clark & H. Nagaishi, unpublished results). In addition both block UV induction of protein X (17; D.F. Spencer, personal communication; M. Volkert and A.J. Clark unpublished results).

In Fig 3 we have compared the abilities of several strains to perform UV-inducible (or activable) reactivation of lambda (R.H. Rothman, L.J. Margossian and A.J. Clark in preparation) and S13 (A.J. Clark and L.J. Margossian, in preparation) with their abilities to perform intrareplicational repair (35). The wild type (uvr^+ $recF^+$) and the uvrA and uvrB single mutants show little difference in either ability. The principal difference is that uvr mutants are induced at lower doses. Addition of recF143 to the uvrA and uvrB mutations blocks reactivation of both phage and blocks intrareplication repair after a dose of $4 J/m^2$. The latter result is virtually identical to that found by Sedgwick (40) for a uvrA6 lexA102 strain. Thus it seems that induced reactivation and intrareplicational repair are totally dependent on recF as they are on recA and lexA.

The fourth column of Fig 3 shows results for the uvr recF143 single mutant. These indicate the occurrence both of intrareplicational repair and some induced reactivation of UV-damaged lambda but no reactivation of UV-damaged S13. In our earlier discussion of intrareplication repair both breakage-reunion gap-filling and transdimer synthesis were pictured as occurring in the absence of dimer excision (Fig 2), and hence were expected to be independent of uvrA and uvrB. Explanation of the uvrA and uvrB dependence may be approached in two ways.

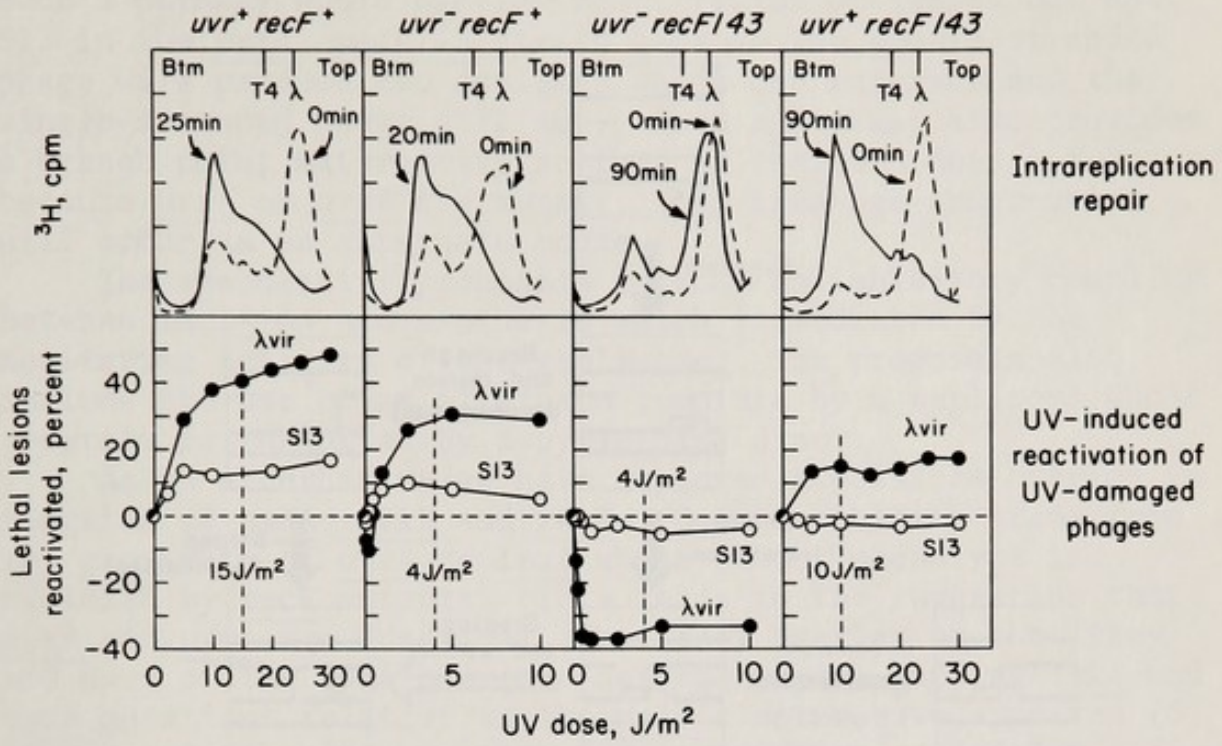


FIGURE 3. Intrareplication repair and UV-induced reactivation of UV damaged phages in uvr and recF mutants. Intrareplication repair data are taken from Rothman and Clark (35). Reactivation data of lambda were taken from R.H. Rothman, L.J. Margossian and A.J. Clark (submitted to PNAS); those of S13 were taken from A.J. Clark and L.J. Margossian (in preparation). Dashed lines on the reactivation curves indicate the doses used to determine intrareplication repair above.

One way has been proposed by Bridges (3). He suggests that uvrA-uvrB dependent excision converts multidimer to monodimer chromosome segments in regions where incomplete replication has left "overlapping gaps". The resulting monodimer segments are repaired by breakage-reunion gap-filling or further excision. The second way is to consider only monodimer segments and propose an excision dependent intrareplicational pathway operative on such segments or an overlapping function for the uvrA, uvrB, and recF genes (35).

COPY-CHOICE EXCISION REPAIR AND A POSSIBLE REGULATORY ROLE FOR uvrA PROTEIN

The excision dependent intrareplicational pathway we envisage involves some highly speculative proposals. The interaction of the two parts of an incompletely replicated segment begins by synapsis forming a D loop (Fig 4). The

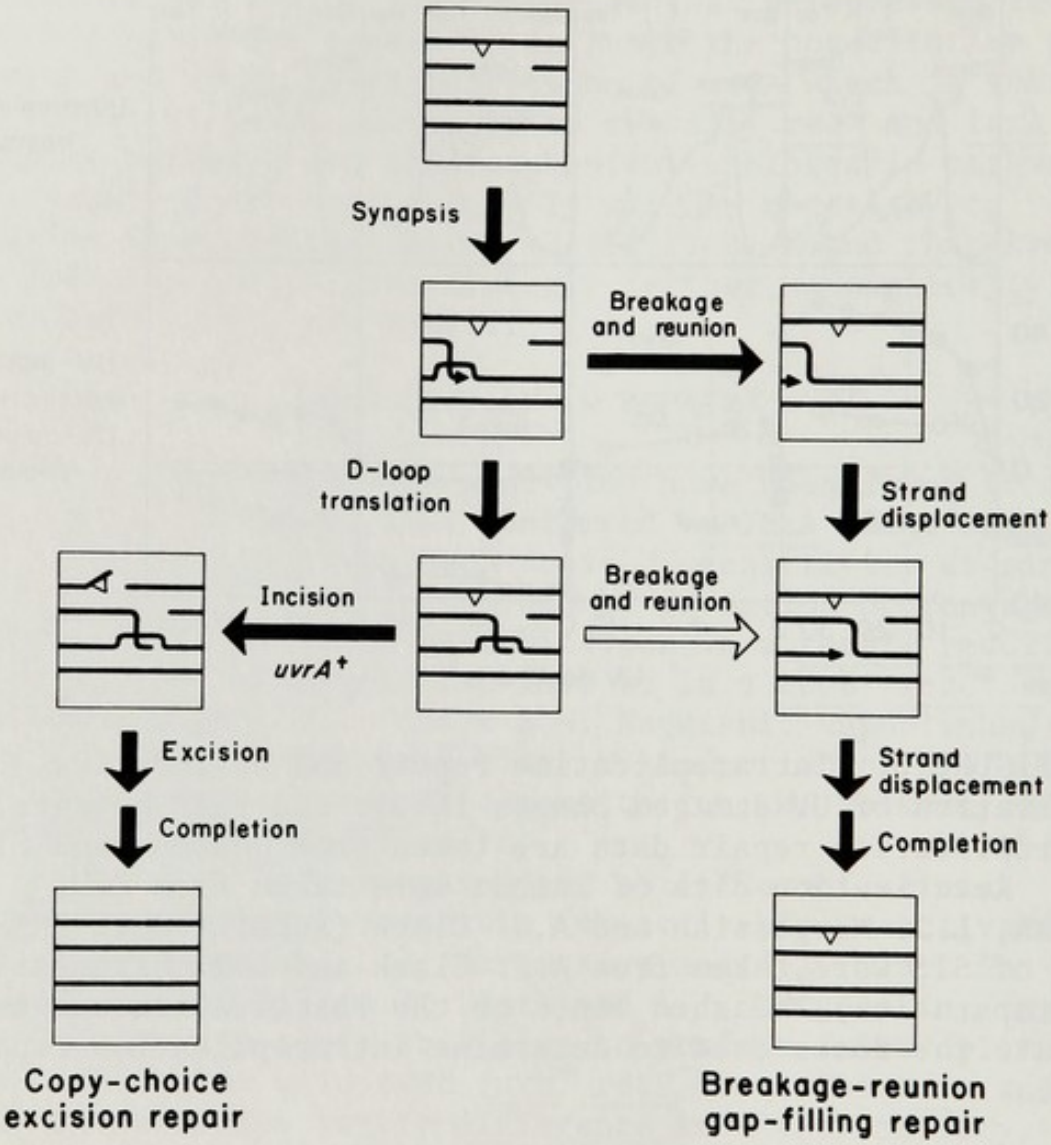


FIGURE 4. Alternative copy-choice excision and breakage-reunion gap-filling pathways of intrareplication repair. Symbols are defined in the legend to Fig 2. The box at the top contains both portions of an incompletely replicated monodimer chromosome segment.

synaptic product is at a branch point between breakage and reunion and continued synthesis from the 3'terminus along the alternate template provided by synapsis which results in D-loop translation. This is accompanied by strand assimilation along the dimer-containing strand. At some point the replication complex (i.e. replisome), which is monitoring the duplex behind and covalently linked to the primer, detects the pyrimidine dimer mismatch. Further forward synthesis then halts while excision repair occurs.

We call the proposed pathway copy-choice excision repair. Such a pathway would provide reactivation of lambda but not S13 in the uvrA+ recF143 strain because the double stranded phage will produce two duplexes which can interact and the single-stranded phage will not. Such a pathway also provides a branch point alternative so that if incision does not occur because uvrA or uvrB are mutant, then breakage and reunion will occur as an alternate route.

The speculative proposals include an inhibitory coupling between incision and synthesis which is mediated by the monitoring activity of the replisome. The proposals also include the use of an alternate template by a replisome whose progress is inhibited by a pyrimidine dimer.

As an alternative we have explored a possible overlap in function of uvrA, uvrB and recF. One possibility stems from the proximity of uvrA to lexA whose mutant phenotype is mimicked by recF mutants. This leads to the suggestion that lexA and uvrA are identical, that they overlap or that they are part of the same operon. Several papers mapping lexA and uvrA mutations relative to the malB cluster have appeared (6, 23,29). In the latest paper (23) a mutation called lex-113 appears to map on the opposite side of uvrA155 from lexA102. Although lex-113 may be in a gene different from lexA (namely lexC) it is also possible that marker effects (e.g. 31) have distorted the results of the two-point crosses employed. Also possible is that uvrA mutations lie within lexA so that uvrA is a gene which overlaps lexA or it represents a cluster of mutations affecting a domain of the LexA protein not affected by the mutations called lexA or lexC.

Another possibility of functional overlap stems from the regulatory differences from wild type shown by uvrA and uvrB single mutants (13). This could be explained by the gene identity, gene overlap or coordinate regulation suggested above or by the interaction of uvrA and lexA products as subunits of a holoenzyme. uvrB mutations might have effects similar to uvrA mutations because of interactions with the uvrA product.

A rationale for the proposed coupled behavior of incision and DNA synthesis on an alternate template stems from the proximity of dnaB to lexA, uvrA and lexC. One study (E. Witkin, personal communication) links dnaB43 very closely with lexA102. A second study (39) puts dnaB on the same side of uvrA as lexA and proposes a hotspot of recombination in dnaB. Such a hotspot might explain the distance between lexA and uvrA mutations should these genes prove to be identical. It is even possible that dnaB is identical to, overlaps with, or is coordinately regulated with lexA, uvrA and lexC. If the genes are not identical their products may interact. Such

possibilities suggest several explanations of the regulatory effects of dnaB mutations and the temperature sensitive DNA synthesis effects of lexC113 mutations. (5,14,48).

MULTIPLE PATHWAYS OF BREAKAGE-REUNION GAP-FILLING REPAIR

An analysis of the genetic dependence of the three intrareplication repair pathways thus far considered reveals the necessity for proposing a fourth. Copy-choice excision is considered a uvrA dependent alternative to recF dependent intrareplication repair. recF143 is seen (Fig 3) to block UV-induced (or activated) reactivation of UV-damaged S13. Since this reactivation is considered to reflect the occurrence of transdimer synthesis this pathway must be recF dependent. recB and recC mutants are UV sensitive and yet these genes are not implicated in short or long patch repair (P. Cooper, personal communication). Consequently we consider breakage-reunion gap-filling to be a recB recC dependent pathway. This accounts genetically for the three pathways and explains the interaction of recB and recF mutations to mimic the sensitivity produced by recA mutations in wild type and uvrA or uvrB single mutant backgrounds (20,35).

We know, however, that the UV sensitivity of a recB mutant can be fully suppressed either by an sbcA or sbcB mutation (1,20). In the suppressed strains the UV resistance is fully recF dependent (20; J.R. Gillen and A.J. Clark, in preparation). As pointed out by Rothman et al (36) there may be a recF dependent pathway of breakage-reunion gap-filling which serves as an alternate for the RecBC pathway. Since this echoes the proposal of separate RecBC and RecF pathways of genetic recombination (7) we have adapted the diagram relating these pathways in our summary diagram (Fig 5).

We suspect that the intrareplicational repair seen following very low doses of UV irradiation to uvrA6 recF143 and uvrB5 recF143 mutants (25,35) is due to the RecBC pathway of breakage-reunion gap-filling. The same phenomenon is observed with a lexA uvrA double mutant (D. George, personal communication) and is a further example of the similarity of lexA and recF mutant phenotypes. It must be explained why this RecBC pathway operates only after very low doses and seems ineffective after higher doses. At present we suspect that the determinative factor may be the amount of protein X available. After very low doses the RecBC pathway may require only constitutive levels of protein X; after higher doses induced levels may be required. Since recF and lexA mutations block UV induction of protein X they may restrict the effectiveness of the RecBC pathway while not blocking it completely.

The lexA mutants that resemble recF mutants are those which are now suspected of rendering the LexA repressor insensitive to degradation by the RecA protease (15,16,28) Other mutations suspected of being in lexA have been isolated whose effects on repair, recovery, and reactivation, need to be accounted for by the pathways suggested (30,46). We find it possible to do this by assuming that these mutations (e.g. tsl and rnm) affect either directly or indirectly uvrA dependent copy-choice excision or recB-recC dependent breakage-reunion gap filling.

SUMMARY AND EVOLUTIONARY PERSPECTIVE

The pathways discussed are summarized in Table 1 and a summary diagram of their interrelationships is presented in Fig 5. It should be emphasized that we consider this analysis to be incomplete and subject to substantial revision when the elements we have omitted are taken into account. The most obvious omission is the repair of DNA segments containing two or more pyrimidine dimers. This is a particularly serious omission because, even after low doses, pyrimidine dimers are distributed in clusters rather than randomly according to the Poisson law (4). We recognize especially that extending the

TABLE I
DNA REPAIR PATHWAYS (new proposals in parenthesis)

		Gene Dependence					
Intrareplication Repair	Pathway	uvrA uvrB	recA	lexA	recF	recB recC	Control
	1. Breakage-reunion gap-filling (RecBC Path)	-	+	-	-	+	Constitutive
Incompletely Replicated Segments	2. Breakage-reunion gap-filling (RecF Path)	-	+	+	+	-	Inducible or Activable
	(3. Copy-choice excision)	(+)	(+)	(?)	(-)	(-)	(Inducible or Activable)
	4. Transdimer synthesis	-	+	+	+	?	Inducible or Activable
Extrareplication Repair							
Unreplicated Segments	1. Short patch excision	+	-	-	-	-	Constitutive
	2. Long patch excision (?)	+	+	?	?	-	Inducible or Activable
Completely Replicated Segments	1. Short patch excision	+	-	-	-	-	Constitutive
	2. Long patch excision	+	+	?	?	-	Inducible or Activable
	(3. Incision-promoted recombination excision)	(+)	(+)	(?)	(?)	(-)	(Inducible or Activable)

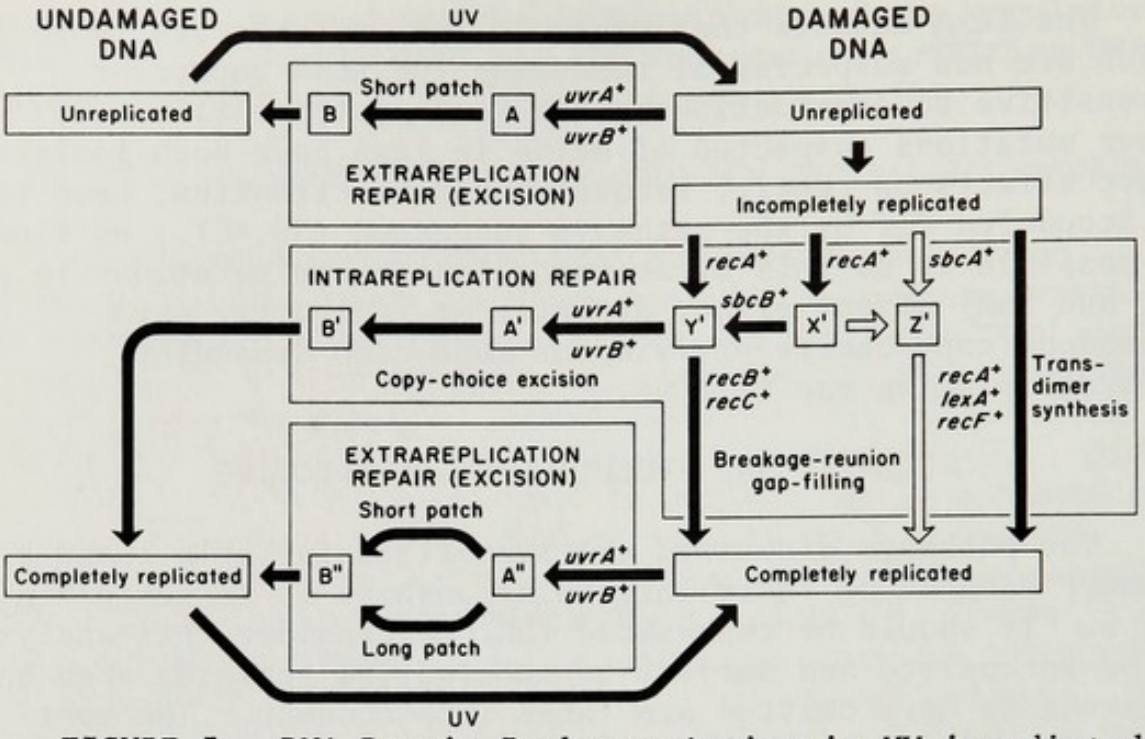


FIGURE 5. DNA Repair Pathways Active in UV irradiated wild type E. coli cells (➞). Undamaged DNA is converted to damaged DNA by irradiation. Excision pathways remove damage; other pathways complete replication of damaged templates. (⇨): pathways effectuated by sbcA⁻ or sbcB⁻. A,A',A",B,B": two classes of excision intermediates. X',Y' and Z': dimer-containing recombination intermediates (7).

analysis to multidimer segments of DNA may obviate the necessity of considering a copy-choice model of excision repair.

The possible identity or overlap of lexA, dnaB and uvrA or the interaction of their products raises the question of the evolutionary relation between replication and repair on one hand and among repair pathways on the other. Some suggestions have already been made (e.g. 18,27) and we would like to add our view. When organisms multiply under continuous UV irradiation, pyrimidine dimers are essentially normal DNA constituents. If primitive conditions on earth favored such a situation it is not clear that extrareplicational repair would have been required for viability. It seems possible that intrareplicational repair pathways were all that was required and that the enzymes of such pathways were constitutive. If extrareplicational repair was a later evolutionary advance, it seems plausible that at least one component would be multifunctional and would regulate the predecessor intrareplicational pathways. In addition it seems plausible that a component of replication or the base sequence determining a normal component of replication would be used to perform both the regulatory function and the extrareplicational

removal of damage. Evidence supportive of this idea may emerge from an investigation of the complete or partial identity of dnaB, the gene determining a normal component of replication (44), lexA, the regulator of recA and presumably of other genes involved in intrareplicational repair, and uvrA, the gene determining one of the components of the incision step which initiates all extrareplicational repair pathways and the interactions of their products.

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MAKING AND CORRECTING ERRORS IN DNA SYNTHESIS : IN VITRO STUDIES OF MUTAGENESIS[1]

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ABSTRACT : Mutagenesis by mis-pairing and by non-pairing DNA lesions is reviewed. Mis-pairing DNA bases, which are readily copied or incorporated into DNA, appear to be subject to a post-replicative methylation-instructed mismatch repair. Non-pairing DNA lesions, usually bulky adducts to the bases, cannot be copied by the constitutive DNA replication enzymes either in vivo or in vitro, and therefore do not result in direct mutagenesis. This is probably due to the polymerase-associated 3' to 5' exonuclease (proof-reading) activity. Such a 3' to 5' exonuclease with typical proof-reading activities has been isolated from mammalian cells. Unrepaired non-pairing DNA lesions cause SOS induction which would seem to result in reversible inhibition of the proof-reading activity, causing decreased replicational fidelity ("untargeted" mutagenesis) and an increased capacity to copy DNA lesions ("targeted" mutagenesis c.f. Witkin, this volume). The observation that unrepaired DNA damage also increases the cellular recombinogenic activity, leads us to speculate that SOS-induction might contribute to the adaptability (population fitness) through rapidly inducible mechanisms of genetic variability e.g. mutagenesis and recombinogenesis.

INTRODUCTION : CONCEPTS AND HYPOTHESES FOR MUTAGENESIS BY MIS-PAIRING AND NON-PAIRING DNA LESIONS

Mutagenesis can occur through at least two principal pathways depending on the nature of DNA lesion (e.g. base modification) involved (1). Mis-pairing DNA lesions (e.g. tautomers and isomers of normal basis deamination products of normal bases, base analogs and oxygen-alkylations of guanine and cytosine) are subtle modifications of DNA bases which are readily (mis)copied or misincorporated by DNA polymerases, depending whether the modified base resides in the DNA template

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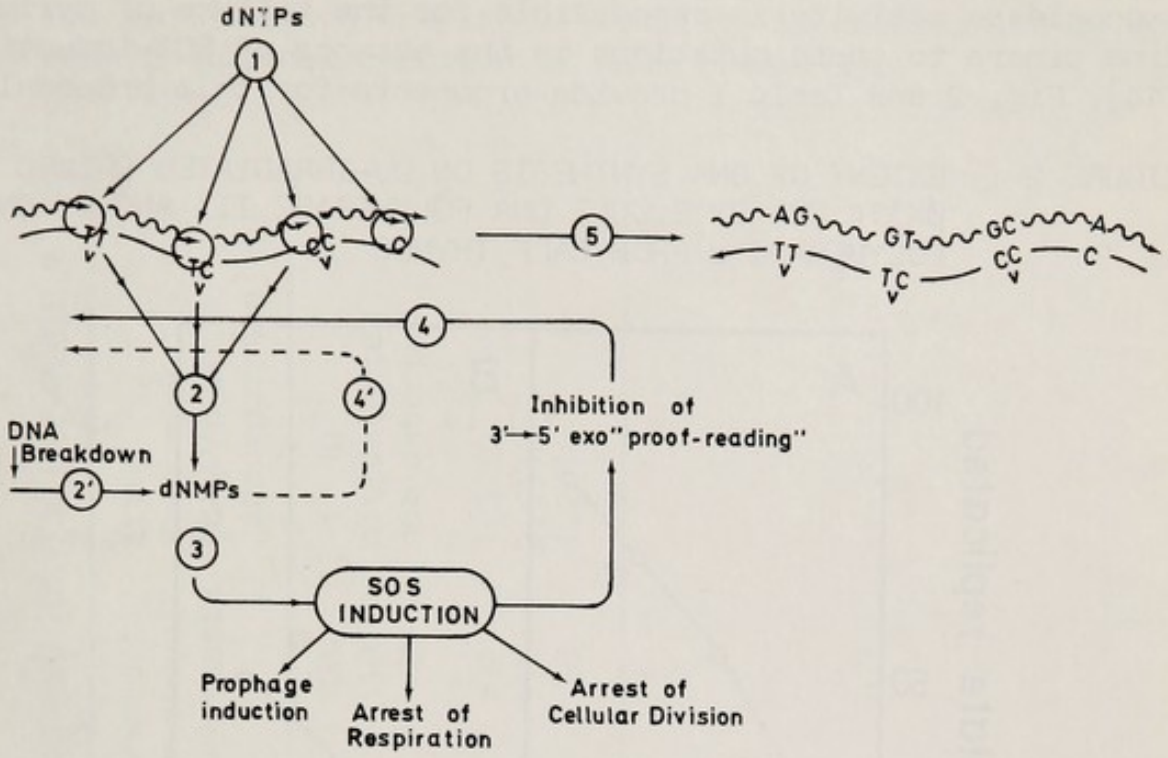
or in the nucleoside triphosphate. Hence a relatively low toxicity and high base change specificity of mispairing mutagens[2]. In vitro studies indicate that microbial DNA polymerases possess a limited capacity to discriminate against stable misincorporation of some base analogs by virtue of the polymerase-associated 3' to 5' exonuclease activity[3] which preferentially excises 3'OH terminal bases, when these are mispaired [4]. However a major error-avoidance mechanism which eliminates misincorporated bases from the newly synthesized strands appears to be a DNA-methylation-instructed mismatch excision repair (Radman, M. Wagner, R. and Meselson M. unpublished experiments). It has recently been found that mutants deficient in generalized methylation of adenine residues (dam)[5] are hypermutable by base analogs and by EMS but not by UV irradiation which produces non-pairing DNA lesions ; this is interpreted as being due to the loss of discrimination between the old and new strands in the mismatch repair[6].

Unlike mispairing lesions, non-pairing DNA lesions are usually bulky base adducts with little or no hydrogen bonding specificity ; therefore these lesions strongly inhibit DNA synthesis and are very toxic. Furthermore, such lesions are not directly mutagenic, since they cannot be copied by normal constitutive DNA replication machinery [7,8]. Non-pairing DNA lesions provoke induction of some otherwise silent genes (SOS-induction)[9 10], whose products are responsible for a temporary acquirement of a cellular mutagenic capacity which is required for the fixation of mutations in the strand opposite non-pairing DNA lesions. The detailed mechanism of SOS-induction is still obscure, inspite of recent discovery that a protease activity is involved in SOS induction [11,12,1] This paper is a review of experimental approaches developed in our laboratory aimed at elucidation of molecular events involved in mutagenesis by UV light. Pyrimidine dimers, which are the most important premutagenic lesions produced by UV irradiation, are typical non-pairing DNA lesions.

Figure 1 represents an attempt to rationalise our current knowledge concerning UV-mutagenesis and to show our working hypothesis as to its mechanism. The central idea, supported by circumstantial evidence [13] is that SOS-repair is an inducible error-prone DNA polymerizing activity, permitting DNA synthesis past pyrimidine dimers and causing incorporation of non-complementary bases both opposite pyrimidine dimers ("targeted" mutagenesis, see Witkin, this volume) and elsewhere in the DNA ("untargeted" mutagenesis).

FIGURE 1 : A HYPOTHETICAL SCHEME FOR UV-INDUCED MUTAGENESIS IN BACTERIA



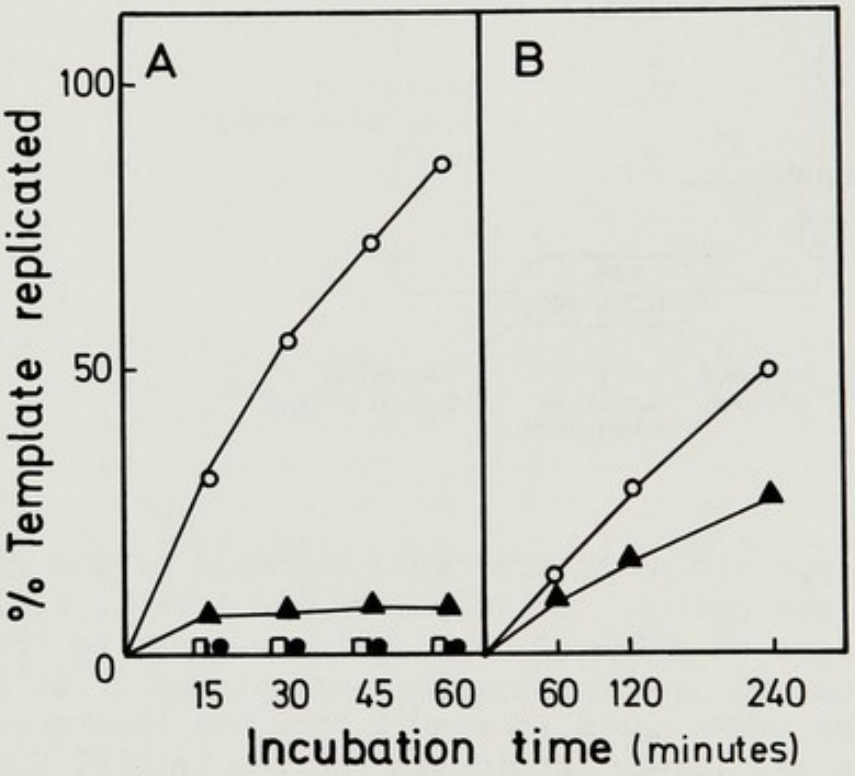
UV induced pyrimidine dimers cause blockage of the elongation of nascent DNA chains, due to polymerase idling, i.e. abortive incorporations of dNTPs (1) and their turnover to free dNMPs (2) caused by the polymerase-associated 3' to 5' exonuclease. Free dNMPs can also result from DNA breakdown (2'), hence dNMPs are likely candidates for the initial SOS-inducing signal (3). Inhibition of the 3' to 5' exonuclease, either as a direct effect of dNMPs (4'), or by an SOS-induced protein (4), would allow DNA synthesis past pyrimidine dimers accompanied by frequent misincorporations of non-complementary bases (5). Frequent tandem base changes opposite pyrimidine doublets have been found during the sequencing of UV induced mutations (J.H.Miller and W.Gilbert, pers.comm. and ref. 2)

THE ROLE OF 3' TO 5' EXONUCLEASE "PROOF READING" FUNCTION IN POLYMERASE IDLING AND IN MUTAGENESIS

The polymerase-associated 3' to 5' exonuclease activity removes mismatched bases from the 3'OH terminus of the primer strand (4), and has been shown to determine spontaneous mutation rates in T4 phage (3 and references therein). We have demonstrated that this activity causes idling of DNA polymerase I due to hydrolysis of dXTPs to free dXMPs in the absence of DNA chain elongation (abortive incorporations) (14 and fig. 1) thereby resulting in the failure of the polymerases to

copy pyrimidine dimers. This dXTP turnover probably due to the absence of Watson-Crick hydrogen bonding of all normal DNA bases with pyrimidine dimers. We have argued that 3' to 5' exonuclease activity is responsible for the failure of pyrimidine dimers to cause mutations in the absence of SOS-induction (14). Fig. 2 and Table I provide arguments for this proposal.

FIGURE 2 : EXTENT OF DNA SYNTHESIS ON UV-IRRADIATED PRIMED ØX174 DNA BY E.COLI DNA POLYMERASE III AND BY DNA POLYMERASE α FROM CALF THYMUS



The construction of the template and reaction conditions have been described (14). Irradiated ØX174 DNA (100 J/m^2) contains about 10 pyrimidine dimers (8). Symbols : (○ — ○) synthesis on intact DNA, (▲ — ▲) synthesis on irradiated DNA, (□, ■) synthesis by ammonium sulfate fraction of the E.coli polA1 mutant, in the presence of 0.2 M KCl and 10 mM N-ethyl-maleamide, inhibitors of DNA polymerase III only. (A) E.coli extract, pol III-dependent synthesis ; (B) DNA polymerase α extensively purified from calf spleen.

DNA polymerase III, the replicative enzyme of E.coli, possesses the 3' to 5' exonuclease activity (15) and is unable to copy pyrimidine dimers in either ØX174 phage DNA (Fig. 2 A) or in poly(dC) templates (Table I). The presence of $\hat{cc}$ dimers in the poly (dC) template does not significantly increase the misincorporation of dAMP (Table I). On the other hand, DNA polymerase α (the presumed mammalian replicase), highly purified and

essentially free of any contaminating exonuclease (Spadari,S. and Villani, G.,unpublished) copies very efficiently both UV irradiated ØX174 DNA (Fig. 2B) and poly(dC) template (Table I) and the synthesis on the latter template is accompanied by an increase in misincorporation of dAMP (Table I).

TABLE I

FIDELITY AND INHIBITION OF DNA SYNTHESIS ON UV-IRRADIATED POLY(dC) TEMPLATE *

Enzymes	UV fluence (J/m^2)	Correct synthesis (picomoles) dGMP	Incorrect synthesis (picomoles) dAMP	Percent residual synthesis	Error frequency (dAMP/dGMP incorporated)
E.coli DNA polymerase III	0	796	0.009	100	$1 \ 10^{-5}$
	10,000	149	not detected	19	not detected
	50,000	17	not detected	2	not detected
Calf spleen DNA polymerase α	0	613	0.10	100	$1.3 \ 10^{-4}$
	10,000	688	0.25	112	$3.6 \ 10^{-4}$
	50,000	159	0.20	26	$1.2 \ 10^{-3}$

* These data are taken from Table III in ref.(14).

Table I also shows that purified E.coli polymerase III shows higher fidelity than the purified DNA polymerase α. This important difference between these two DNA replicating enzymes does not tell us much about the mutagenic rates and mechanisms in E.coli and mammalian cells respectively (see below), but it does indicate a crucial role of 3' to 5' exonuclease in the efficiency and fidelity of in vitro DNA synthesis on UV-irradiated templates. This and other findings (see below) lead us to propose that SOS-induction may involve synthesis of an inhibitor of 3' to 5' exonuclease (Fig. 1 and ref.1), and that consequently, SOS repair may be accounted by an inducible DNA synthesis permitting chain elongation past otherwise non-reparable pyrimidine dimers (increased survival), at the expense of replicational fidelity (mutagenesis).

TESTING THE HYPOTHESES : SOME NEGATIVE RESULTS

1) No Evidence for the Induction of a New DNA Polymerase. Using an E.coli polA⁻ polB⁻ polCts mutant, UV-irradiated or not, we could find no evidence of a new DNA polymerase activity, tested at 42°C, from irradiated cells. Furthermore, 600 r X-ray irradiation of C3H 10T 1/2 mouse fibroblast cells, harvested at 0, 5, 15 and 20 hours after irradiation, caused no detectable induction of either deoxyribonucleotidyl terminal transferase (TdT) nor of the reverse transcriptase-like enzymes (specific radioactivity of dXTPs was 5000 cpm/pmol).

2) The Failure to Purify the "Error-Prone DNA Polymerase" Activity. Crude extracts from SOS-induced E.coli (using tif-1 mutant and a mutator plasmid pKM101) often show increased misincorporation rates on poly pyrimidine homopolymers (13 and unpubl.expt.). Our attempts to purify the low fidelity DNA polymerase have failed ; the fidelity of DNA polymerases recovered from column chromatography was the same for induced and non-induced extracts, suggesting either no direct modification or an unstable modification of DNA polymerases.

3) The Sedimentation Properties of DNA Polymerases I and III from SOS-Induced E.coli. These analyses were performed to test the possibility of a proteolytic cleavage of DNA polymerases in the course of SOS-mutagenesis. Using tif-1 mutant at 42°C as the inducing treatment, no evidence was found for a decrease in molecular weight of DNA polymerases I and III.

4) No Effect of the recA (tif-1) Protein on the Fidelity of E.coli DNA Polymerase III. Fraction V of E.coli DNA polymerase III (15) was pre-incubated with 100 µg/ml purified E.coli recA (tif-1) mutant protein (gift of

Dr. J. Roberts) for 120 min at 37°C under the conditions of inactivation and and proteolytic cleavage of λ repressor [17] Subsequently, substrates were added to test for polymerase fidelity as in Table I. The pre-incubation inactivated only 40 % of polymerase III activity, but did not change its fidelity (error frequency dAMP/dGMP with poly(dC) as template was 5×10^{-5}).

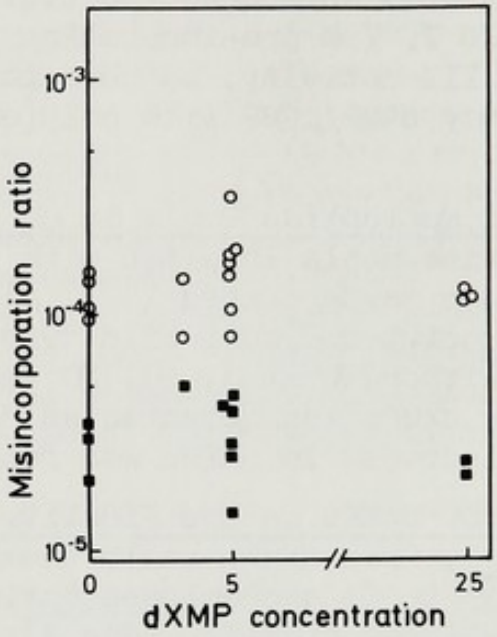
5) Analysis of Nucleotide Pools in UV Irradiated E.coli. Analysis of nucleotide pools labelled with ^{32}P for two generations was performed as described [17]. Aliquots from unirradiated and UV irradiated E.coli wild type, uvrA$^-$, recB$^-$ and uvrA$^-$recB$^-$C$^-$ were withdrawn at 0, 10, 20 and 30 min. No UV-induced increase in dXMPs was detected as expected from Fig.1, but a significant increase in dXTPs was repeatedly observed.

6) The Effect of dXMPs on the Fidelity of E.coli DNA polymerase I. Free dXMPs could themselves lead to the inhibition of the 3' to 5' exonuclease activity, and hence to a decreased replicational fidelity (Fig.1). Furthermore, Hopfield's kinetic proof-reading scheme [18] predicts that dXMPs could be directly incorporated without kinetic proof-reading. The excess of dXMPs (or of any single dXMP, results not shown) did not cause any consistent, dose-dependent effect on the extent and fidelity of DNA synthesis by E.coli DNA polymerase I on poly(dC) template (Fig.3). These results are in agreement with those from Dr. Loeb's Laboratory (L. Loeb, priv. commun.) and in disagreement with ref.19, but the latter may be due to the important difference in the measures of replicational fidelity (comp. Table III, ref.19 and Table I, this paper).

AN EVIDENCE FOR THE EXISTENCE OF A PROOF-READING ENZYME IN MAMMALIAN CELLS

Several observations lead us to postulate the existence of a proof-reading 3' to 5' exonuclease in mammalian cells : (1) the fidelity of DNA polymerase α decreases with the degree of purification and this can be correlated with the loss of a contaminating 3' to 5' exonuclease (20), (2) the capacity of DNA polymerase α to copy UV-irradiated ØX174 DNA increases with the degree of purification [20]. Since DNA synthesis in mammalian cells stops at each pyrimidine dimer [21], the latter observation suggests the presence in vivo and in crude extracts of factor(s) which prevent DNA synthesis past pyrimidine dimers, perhaps a 3' to 5' exonuclease. Table II shows the properties of the 3' to 5' exonuclease purified from calf spleen (Spadari, Villani and Radman, in preparation)

FIGURE 3 : EFFECT OF DEOXYRIBONUCLEOSIDE MONOPHOSPHATES ON THE FIDELITY OF E.COLI DNA POLYMERASE I.



Purified E.coli DNA polymerase I, poly(dC):oligo(dG), reaction and assay conditions were described elsewhere (14). dXMP was an equimolar mixture of dAMP, dGMP, dTMP and dCMP. Abscissa indicates mM concentration of dXMPs. Open circles are values for the ratio dAMP/dGMP incorporated, while closed squares are values for the dTMP/dGMP ratio. The difference indicates that tranversion errors are less frequent than transition errors. Each point represents an independent fidelity assay.

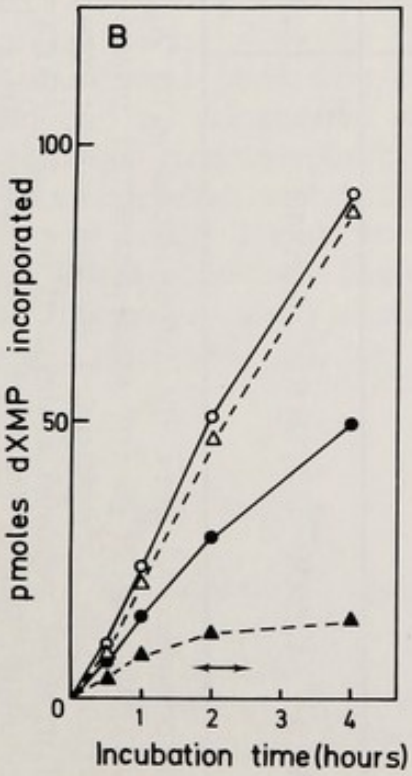
TABLE II

ENZYMATIC PROPERTIES OF THE 3' TO 5' EXONUCLEASE FROM CALF SPLEEN

1. Cation requirement : Mg^{++}or Mn^{++}, optimum 3mM for Mg^{++}
2. pH optimum : about 9 in 50 mM glycine-NaOH buffer
3. Exonucleolytic activity : exclusively in the 3' to 5' direction ; releases only dXMPs.
4. Specificity : attacks both double-stranded and single-stranded DNA, but under competitive conditions degrades preferentially single-stranded DNA. Mismatched bases are excised faster than matched bases.
5. Sedimentation coefficient : $S_{W,20}$ is approximately 5.3
6. Approximate molecular weight is 10^5 daltons (from both $S_{W,20}$ and from Sephadex filtration).
7. Intracellular location : both nucleus and cytoplasm ; possible association with DNA polymerase α.

Fig. 4 demonstrates that this enzyme inhibits DNA synthesis by purified calf spleen DNA polymerase α on UV irradiated ØX174 DNA, but not on unirradiated ØX174 DNA. Furthermore, this purified enzyme performs typical proof-reading function (e.g. excision of terminal mismatches as the prerequisite for chain elongation) for DNA polymerase α in the Brutlag-Kornberg type of experiment (4). If this 3' to 5' exonuclease is the real proof-reading enzyme in mammalian cells, which determines the replicational fidelity and which prevents DNA chain elongation past non-coding DNA lesions (Fig.4), then the basic molecular events involved in mutagenesis of mammalian genome might be similar to those in E.coli : an inducible process might be required (22) to permitt mis-copying of DNA lesions e.g. by inactivation of the proof-reading function (Fig.1)

FIGURE 4 : A PURIFIED MAMMALIAN 3' TO 5' EXONUCLEASE INHIBITS SYNTHESIS BY DNA POLYMERASE α ON UV-IRRADIATED ØX174 DNA.



Both enzymes were highly purified from calf spleen (Spadari and Villani, unpublished). The addition of the 3' to 5' exonuclease (see Table II) (Δ - - Δ) did not change the extent of DNA synthesis on intact ØX174 DNA (o – o), however the synthesis on UV-irradiated (100J/m^2) DNA (• – •) was inhibited by the same amount of 3' to 5' exonuclease (▲ - - ▲). Arrow indicates the level of synthesis by E.coli polymerase I on the same irradiated DNA.

TABLE III

RECOMBINATION AND MUTATION FREQUENCIES DURING THE GENETIC CROSSES BETWEEN λc^+ Nam7am53 AND λc^+ Pam80 IN IRRADIATED AND UNIRRADIATED HOST CELLS.

Events analysed	Frequencies from the crosses in C_{600} W.T.				Frequencies from the crosses in AB2494lexA⁻			
	B(o)/ λ(o)	B(UV)/ λ(o)	B(o)/ λ(UV)	B(UV)/ λ(UV)	B(o)/ λ(o)	B(UV)/ λ(o)	B(o)/ λ(UV)	B(UV)/ λ(UV)
Recombination frequency between λ N and P markers	2.9×10^{-3}	9.2×10^{-3}	5.1×10^{-2}	5.6×10^{-2}	1.5×10^{-3}	5.8×10^{-3}	4.0×10^{-2}	3.3×10^{-2}
Frequency of clear plaque mutants	1.3×10^{-4}	4.0×10^{-4}	2.0×10^{-4}	6.6×10^{-4}	1.1×10^{-4}	1.3×10^{-4}	1.1×10^{-4}	2.1×10^{-4}

Bacteria (B) and/or phages (λ) were UV irradiated with 70 J/m^2 and 200 J/m^2 respectively prior to infection at multiplicity of 5 for each parental phage. Each cross was terminated by addition of $CHCl_3$ 2 hours following infection. Lysates were titrated on N 100 (Su^-, $recA^-$) indicator bacteria to score for N^+P^+ recombinants and on C_{600} ($SuII^+$) for the total progeny. Clear plaque mutants were scored using NO483 ($SuIII^+$) indicator which only permits growth of clear mutants. "O" and "UV" indicate intact and irradiated bacteria (B) or phage (λ) respectively. This work will be published by Zambrano and Radman.

INDUCTION OF GENETIC VARIABILITY : AN EVOLUTIONARY ROLE FOR SOS INDUCTION ?

The contribution of the inducible, mutagenic DNA repair to cellular survival is significant but small [10]. However, SOS induction could provide a major contribution to the long-term survival of a population by increasing its adaptive capacity (fittness) e.g. by the rapid increase in genetic variability. For example, X-ray irradiated population of Drosophila shows a higher fittness than the unirradiated control population [23]. X rays are known to increase genetic variability in Drosophila by inducing both mutations and recombination. M. Fox (pers. commun.) and ourselves (Table III) have independently observed that the frequency of genetic recombination of intact phage λ genomes increases if the host cell has been UV irradiated prior phage infection. This effect is independent of the lex gene of E.coli, which is required for induction of the mutagenic capacity (Table III), hence the pathways of induction of the cellular mutagenic and recombinogenic activity are not identical. Evidence has been found in yeast [24], Ustilago [25] and in mammalian cells (Kinsella et al, this volume) that the cellular recombinogenic activities can be induced by mutagenic and/or carcinogenic treatments. These findings suggest that SOS induction may be an ubiquitous genetic programme providing for a rapid increase in genetic variability through induction of mutagenesis and recombinogenesis. These genetic processes lead to the increased population fittness, when a population is exposed to high selective pressure, such as due to unrepaired DNA lesions.

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CELL SURVIVAL, EXCISION REPAIR AND DNA REPLICATION IN EUKARYOTIC CELLS[1]

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ABSTRACT Excision repair is a major set of pathways by which damage is removed from DNA; mutations that affect excision result in large increases (5-10 fold) in UV sensitivity in mammalian cells. Mutations that affect excision-repair indirectly, or that affect the cell cycle, DNA chain growth, replicon organization, etc., may result in smaller increases (2-4 fold) in UV sensitivity. The rate of excision repair depends on the nature and location of damaged sites and their relationship to chromatin structure and the functional state of DNA. Excision of damage involves nicks which relax the supercoiling of DNA and may prevent replicon initiation, thus indirectly affecting the rate of DNA replication. Perturbations in replication are coupled to the cell's excision-repair capacity and "post-replication repair" may merely be a descriptive term for these perturbations and not a discrete repair system.

INTRODUCTION

Xeroderma pigmentosum (XP) was first described as a mutation affecting the initial step of excision repair of UV damage in human cells about 10 years ago (1-3). Although we still generally believe this statement to be true, there is some uncertainty as to how many of the several groups of XP it describes, which of the several branches of excision repair

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we should consider and what constitutes the initial step and its regulation (4). In analysing radiation induced changes in normal and mutant cells we need to distinguish between primary or major effects of damage and secondary effects resulting from interactions between damage, repair and normal cell functions. For example, increased radiation sensitivity may not necessarily mean a cell type is repair deficient, and transient radiation induced changes in DNA synthesis do not necessarily indicate existence of a repair process associated with replication (post-replication repair).

Sensitivity of Various Cell Types to Radiation. Fibroblasts from a variety of human diseases show increased sensitivity to UV, X Rays and some chemical carcinogens (4,5). Once, such changes in sensitivity might have been interpreted using target theory (6); now, the fashion is to use concepts of repair. With no biochemical or other additional evidence, however, the increases alone do not indicate defects in repair, changes in target size, shape or number, or any other cellular characteristic. When there is a clear repair defect (e.g. XP), the sensitivity of fibroblasts is increased 5 to 10 fold (4). The sensitivity of Fanconi's anemia (FA) cells to DNA crosslinking agents is similarly increased nearly 10 fold (7). In other diseases, however, only a 1.5 to 4 fold increase is found and more subtle mechanisms may be involved than simple repair deficiencies (5). XP variant cells for example have slight increases in sensitivity to killing by UV light, but its sensitivity to mutagenic effects of UV light is as great as other XP cell lines (8); this disease might therefore have an excision repair system that makes a high frequency of errors. Progeria is slightly sensitive (9), accumulates a high frequency of altered proteins in culture (10), and increased sensitivity may result indirectly. Bloom's syndrome has a reduced DNA chain growth rate (11) and an abnormal cell cycle (12); both of these changes may have a secondary effect on sensitivity because of variation in sensitivity around the cell cycle and coupling between replication and repair (see below). Down's syndrome, and possibly other chromosomal abnormalities has an altered organization of DNA replication (13) and this may have a secondary effect on sensitivity. Consideration of secondary influences on sensitivity should perhaps be entertained whenever relatively small increases in sensitivity are found.

Excision Repair: Multiple Pathways and Their Regulation. Studies of UV damage and repair have led to the familiar model of nucleotide excision repair, which involves sequential action of damage specific endonucleases, exonuclease, polymerase and ligase. This pathway replaces UV and carcinogen damaged

sites by patches up to 100 bases in length (14). The kinetics of repair replication (15), strand breakage (16) and excision of endonuclease sensitive sites (17) give a consistent picture of repair events occurring progressively through the genome, a few sites at a time.

The initial steps of excision are much more complex than merely the action of one or two enzymes. These steps are affected by each of at least 6 distinct complementation groups in XP (4, Bootsma, personal communication 1977) none of which appear to be simple enzyme deficiencies (21). The sites and rates of repair appear to be modulated by chromatin structure (22,23) and repair may occur more rapidly in some regions of DNA than others, depending on the local state and functional activity of the chromatin (e.g. internucleosomal DNA, regions of transcription, replication, etc. (Fig. 1)).

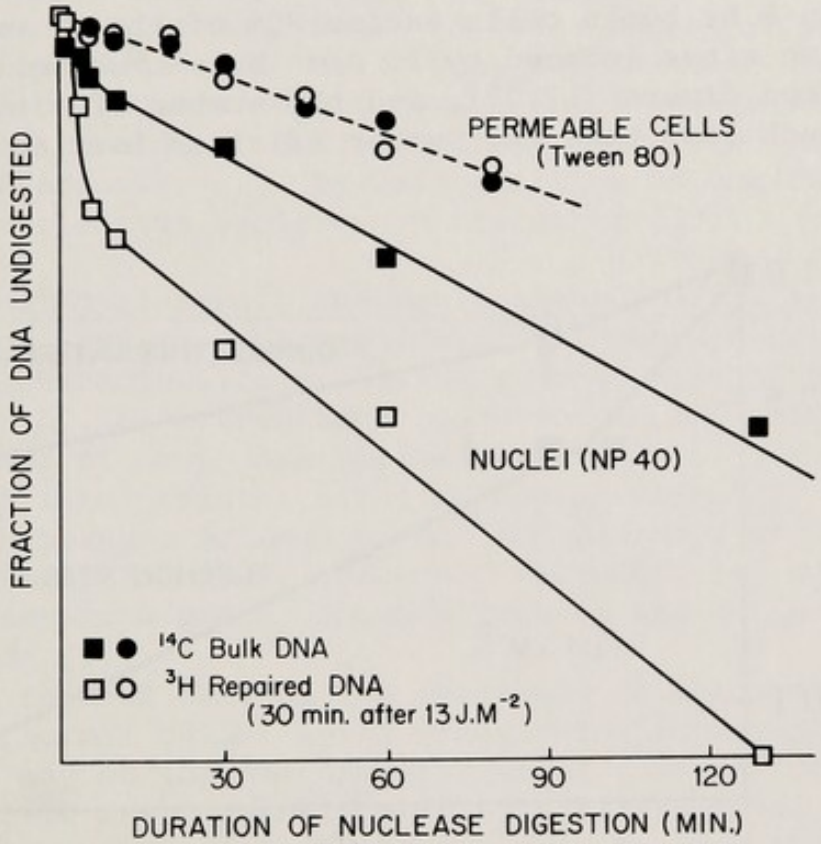

FIGURE 1. Rate of degradation by Micrococcal nuclease of ^{14}CTdR- uniformly labeled DNA (● , ■) and ^{3}HTdR-repaired DNA (○ , □) in human fibroblasts grown for 2 or more days in 0.1 µCi/ml ^{14}CTdR, irradiated with 13 J/m^2 UV light and labeled for 20-30 min in ^{3}HTdR, 10 µCi/ml, 50 Ci/mmole. (● , ○) Cells swollen in hypotonic saline, treated with 1% triton X-100 and dounced extensively to isolate nuclei before enzyme digestion. Nuclei appear to loose components which make repaired regions sensitive to enzyme attack (23).

(■ , □) Cells swollen in hypotonic saline treated with 1% Tween 80 to permeabilize cell membrane. Cells retain more nuclear and cytoplasmic components than in A, and enzyme digestion does not discriminate between ^{14}C and ^{3}H labeled DNA.

Initiation of repair does not appear to require a supercoiled configuration of DNA, as does initiation of semiconservative replication, because X Ray induced breaks in DNA have no effect on subsequent UV-induced repair but preferentially inhibit replicon initiation (24,25). Nicks introduced during excision also cause a relaxation of the supercoiling of DNA at molecular (18,19) and cytological levels (20) and may similarly inhibit replicon initiations.

Excision is apparently slower when measured by chromatography in acid-fixed cells than when measured by prokaryotic UV-endonucleases in high molecular weight DNA (26,27) (Fig. 2). This effect is most marked at early times after irradiation; within 6 hr human cells excise 30% of the UV endonuclease sensitive sites induced by 10 J/m^2 but no chromatographically detected dimers (17,27), and the number of single strand breaks is much less than the number of sites lost (16,28).

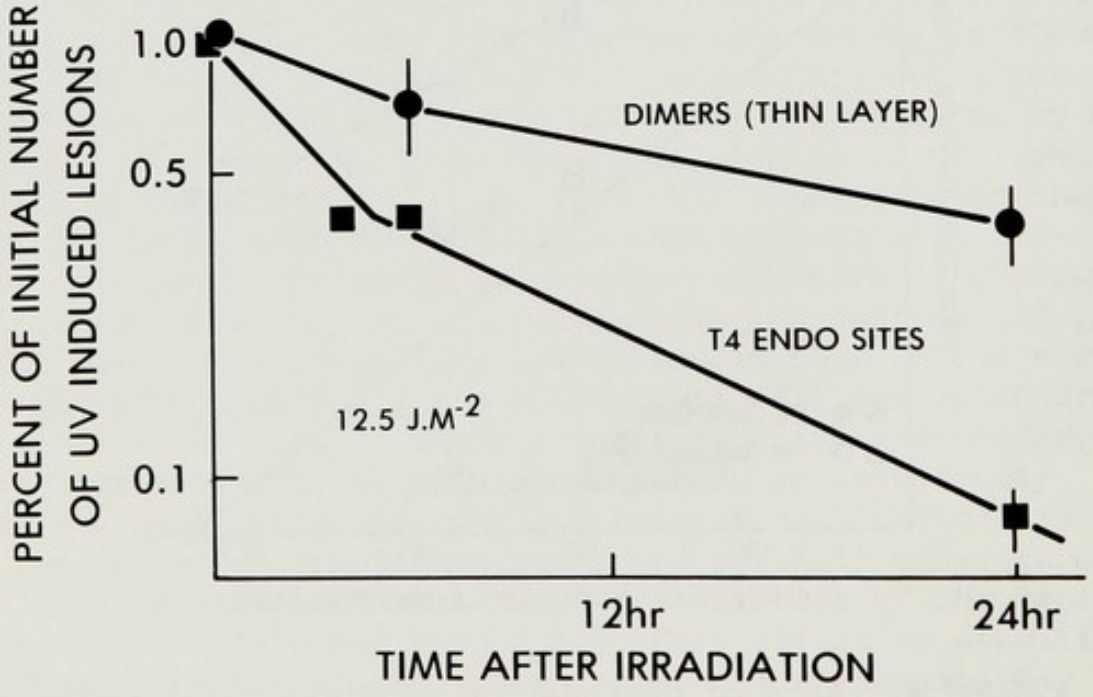

FIGURE 2. Fraction of starting number of pyrimidine dimers (● , 1 dimensional thin layer chromatography) or T4 UV endonuclease sensitive sites (■ , alkaline sucrose gradients) in CV1 monkey cells irradiated with 13 J/m^2, and grown for various times. (data taken from 27).

This apparent inconsistency can be resolved if excised fragments are initially acid-insoluble, but these have yet to be detected and more complex interpretations may be necessary. A related dilemma is found in XP group D cells which do not excise dimers or endonuclease sensitive sites, make no nicks in DNA, but nevertheless perform up to 50% of the repair replication seen in normal cells (4,16,17,29).

Several other pathways of excision repair have recently been discovered. Base excision repair is a pathway by which damaged bases (e.g., alkylated purine, thermal damage) are removed by an N-glycosylase, the strand cleaved by apurinic/apyrimidinic endonucleases and a relatively small patch of a few bases inserted (30,31). It is conceivable that the excised base could also be replaced directly (by a transferase) without cleavage of the polynucleotide strand (Friedberg, personal communication 1977).

DNA-DNA crosslink repair may involve a combination of base and nucleotide excision. FA cells cannot unhook a chemical crosslink whereas XP cells can, but FA cells can excise the corresponding monoadduct damage and XP cells cannot (7, 32). Opposite ends of DNA crosslinks therefore seem to be repaired differently, e.g. by base excision on one strand followed by nucleotide excision on the other (33).

Coupling of Repair and Replication (Definition of Post-Replication Repair. DNA replication in damaged cells has a number of characteristics: (a) a reduced rate of DNA synthesis per cell, (b) a transient reduction in the molecular weights (MW) of newly synthesized DNA, and (c) linkage of the low MW DNA into parental sized molecules (Fig. 3). Although apparent exchanges between parent and daughter strands have been reported (34,35,36) these may represent end addition of newly synthesized DNA to damaged strands and not actual recombination (37).

The reduced rate of DNA synthesis is the predominant response in normal cells, being greater and occupying a longer time than any of the MW changes. About 5 hr or more after UV doses of 5-10 J/m^2, normal cells synthesize normal sized DNA although the rate of synthesis per cell is less than half normal (Fig. 3), implying that less than half the normal number of replicons are active. The reason for this is probably that excision repair breaks have the same effect as X Ray and other DNA breaks (24) in relaxing DNA supercoiling and thereby prevent replicon initiation.

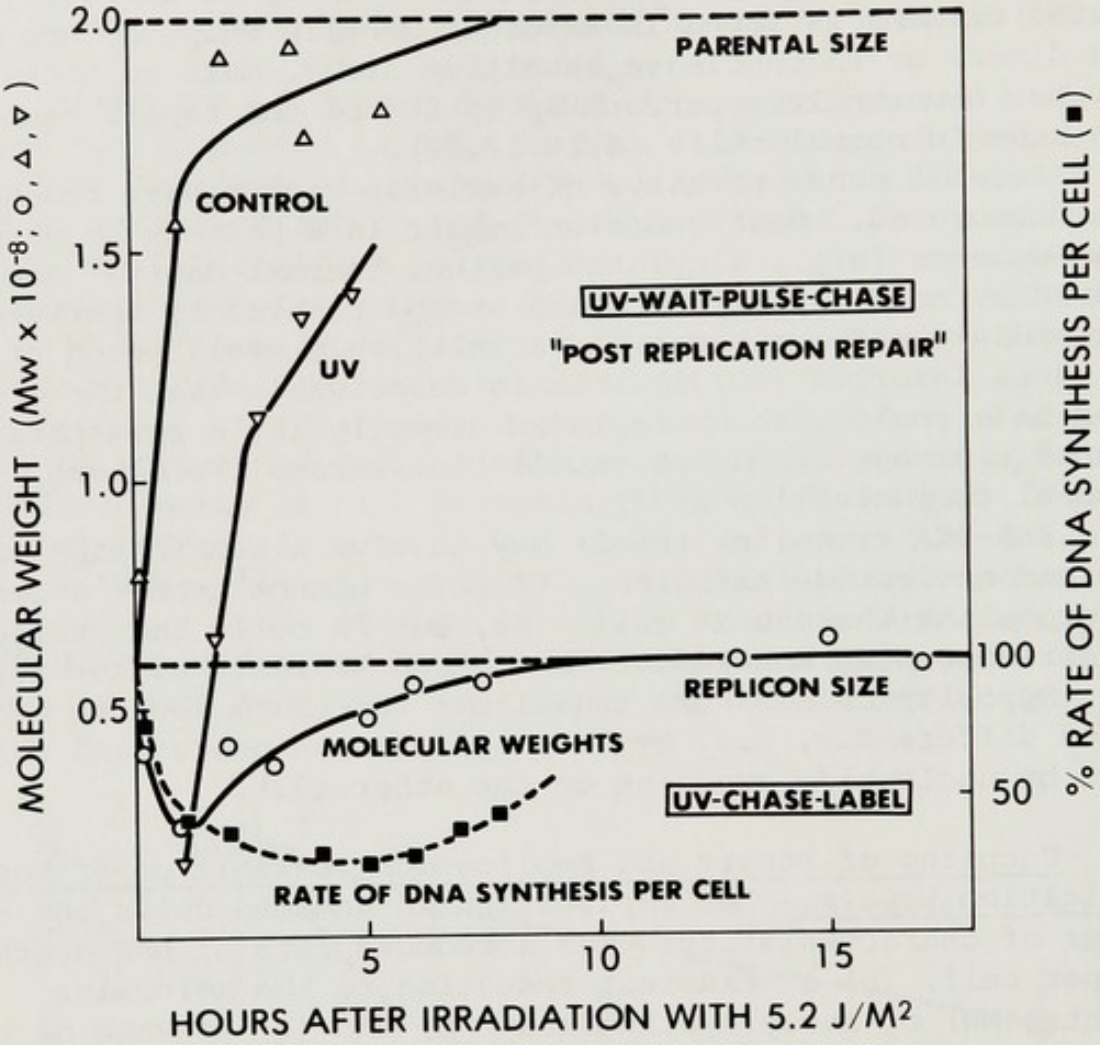

FIGURE 3. Molecular weight changes in human (SV40 transformed) fibroblasts labeled for 10 min with ^{3}HTdR (10 μCi/ml, 50 Ci/mmole) at various times after irradiation with 5.2 J/m^2 UV light and chased for various times. ■ rate of DNA synthesis per cell, ○ weight average molecular weight (M_w) of DNA synthesized at various times after irradiation, △ M_w of DNA in control cells labeled at 0 time and grown for various times, ▽ M_w of DNA synthesized in irradiated cells labeled 1 hr after irradiation and grown for various times.

When actively replicating replicons are damaged, their response is to synthesize DNA in smaller fragments (Fig. 3), possibly because chain growth is blocked by damaged sites. Associated with this block is an increase in the amount of single strandedness (38,39), which might be due to continuation of part of the replication machinery which causes local

denaturation ahead of replicating forks. Cells recover their ability to synthesize normal size fragments as a function of time according to the empirical relationship:

$$M(t) = \left[1 - \left(1 - \frac{M_O}{M_R}\right) \exp - kt\right] M_R$$

where $M(t)$ = single strand MW synthesized in a brief pulse (10-15 min) at t hr after irradiation.
M_O = MW obtained by extrapolation to t = 0
M_R = single strand MW in control cells.
K = a constant describing the rate of recovery, which depends on both repair and organization of replicons.

M_O is approximately equal to the single strand distance between dimers. M(t) always exceeds this value because of the onset of replication in undamaged or mended replicons, or replication around some dimers (39). The extent to which M(t) is reduced depends on the number of damaged sites per replicon (i.e., the ratio of M_R:M_O). Greater reductions in M(t) are therefore seen in those cell types with larger average replicons (e.g. mouse and hamster) than smaller (e.g. human) (40). The reduction in MW seen in excision defective XP and XP variant cells are greater than in normal cells indicating that these reductions are also a function of the repair capacity of the cell. The changes in DNA synthesis in all XP cells are viewed here as secondary consequences of interactions between replication and repair rather than primary defects in a post-replication repair process (31,41).

Linkage of the low MW DNA into near parental sized molecules in normal (Fig. 3), excision defective and XP variant cells (41) proceeds at the same rate as in unirradiated cells, despite starting from a reduced value. This increase in MW has been thought to involve bypass of damaged sites and represent a specific post-replication repair process (41-43). But since its rate is independent of irradiation and of the excision-repair capacity of the cell it may only involve chain elongation processes that operate normally in a cell. There may in fact be no such thing as post-replication repair. There are some unique features, however, about the replication process in irradiated cells because it is altered by caffeine (41,43), but detailed quantitative analysis of its effect is not yet available.

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ELECTRON MICROSCOPY OF UV-INDUCED POST REPLICATION REPAIR DAUGHTER STRAND GAPS[1]

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ABSTRACT Single strand DNA specific gene 32 protein stain and the aqueous spreading technique with cytochrome C allowed observation by electron microscopy of the probable ultraviolet light induced postreplication repair intermediate DNA species from Escherichia coli. Gap size ranged from 1,500 to 40,000 nucleotides.

INTRODUCTION

The average size of daughter strand gaps generated as secondary ultraviolet light (UV) induced lesions in Escherichia coli has been estimated to be 10^3 to 10^4 nucleotides long. Procedures used to determine gap size have included BND cellulose chromatography (1) and dBrUrd photolysis (2). These methods cannot discriminate between true gap structures and double strand DNA with single strand ends and/or gaps in intermediate stages of filling by recombination of DNA. Studies of Takahashi (3) have demonstrated the use of electron microscopy to observe UV enhanced recombination between phage DNA molecules. Mature isolated phage were exposed to UV previous to infection. Use of phage with deficiencies in replication allowed observation of joint recombinant molecules independent of DNA replication structures. In our paper, different methods were employed to maximize and concentrate a different type of recombinant prone DNA species, the post-replication repair intermediate. Cells were allowed to replicate after exposure to UV. An excision deficient mutant of Escherichia coli was used to maximize the number of primary lesions for 6.0 J/m^2 of UV that would plass through the replication fork, and thus increase the yield of daughter strand gaps. The BND-cellulose column was used to separate double strand DNA from double strand DNA with single strand regions, and repeated chromatography concentrated the DNA species of interest. Since strand DNA specific staining with gene 32 protein and the use of a single strand circular DNA standard (ϕ×174 phage DNA) were used to identify the gap structure and

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estimate length. The fine structure of DNA fragments and their respective frequency generated by incomplete postreplication repair are described.

METHODS

DNA Preparation. The bacterial strain E. coli C thy321 uvrA6(4) was obtained from R. Hewitt. DNA fragments with single strand regions were selected by BND-cellulose chromatography (1,5) from E. coli given a 15 minute reincubation after 6.0 J/m^2 UV exposure (6). In some experiments DNA was observed without preselection by chromatography. DNA was phenol extracted and the aqueous suspension was dialyzed extensively against the NaCl-Tris-EDTA buffer. The dialyzed DNA was subsequently treated with ribonuclease and again phenol extracted and dialyzed. DNA applied to the column was sheared by passage through a 23G needle. DNA was prepared for electron microscopy (EM) by the method of Davis et al. (7). $\phi\times174$ DNA was added (0.8 µg/ml). Gene 32 protein was complexed to single strand DNA according to the gene 32-EtBr method of Wu and Davidson(8).

Electron microscopy. Electron micrographs were taken at 60KV with a Phillips 301 electron microscope with 15,000 and 45,000 magnification.

RESULTS

Electron Microscopy of BND-cellulose Selected DNA Fragments Stained by Gene 32 Protein. DNA fragments were released from cells exposed to UV and allowed to reincubate. Previous studies indicate this protocol results in UV secondary lesions described as postreplication repair daughter strand gaps. We have used BND-cellulose column chromatography to separate double strand DNA from double strand DNA with single strand regions. Gene 32 protein staining is specific for single strand DNA, allowing visual confirmation of the proposed gap structure. Gene 32 protein staining of the $\phi\times174$ single strand circular DNA results in a relatively thick DNA-protein complex (Figure 1). The same large diameter of a portion of the E. coli DNA indicates the presence of a single strand region in a double strand DNA fragment.

Electron Microscopy of Single Strand Regions from Fragments not Preselected by Chromatography, and Stained by the Aqueous Spreading Technique. DNA specimens prepared by the aqueous spreading technique of Davis et al. (7) also indicated double strand DNA with a single strand region. Gaps such as seen in Figure 2 were observed in DNA fragments from cells lysed directly before DNA spreading, with no column

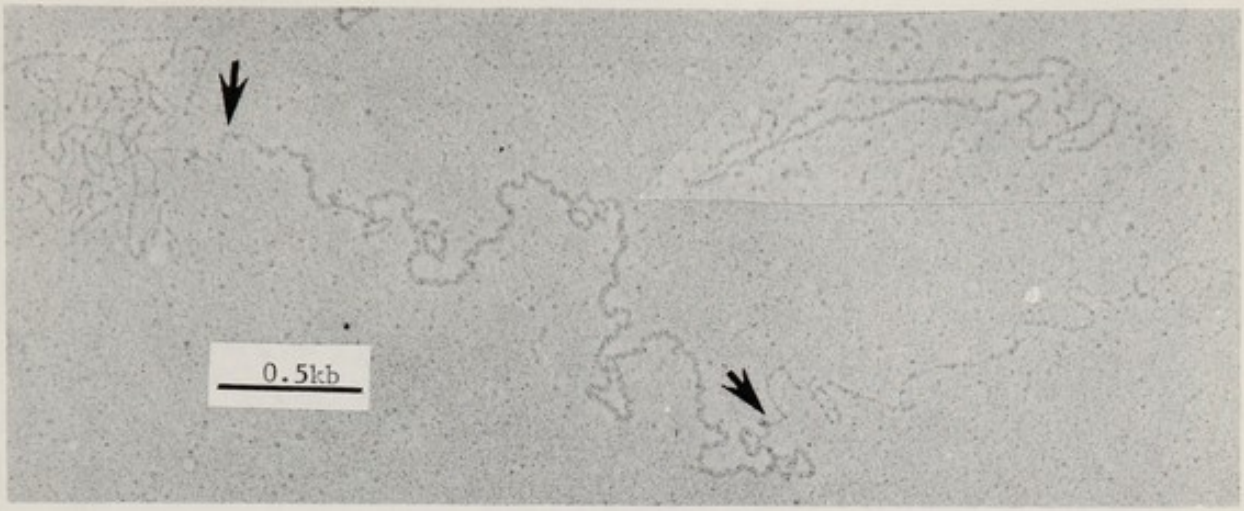

Figure 1. Electron micrograph of a DNA fragment from E. coli including the probable UV induced postreplication repair intermediate daughter strand gap. The ϕ×174 insert was photographed in the same field at the same magnification.

preselection. Gaps were less frequent but larger in samples that were not preselected by chromatography. In contrast to the gene 32EtBr method, the aqueous spreading technique demonstrates the single strand DNA as a less rigid, irregular-strand as opposed to the more rigid double strand DNA. Gap size ranged from 1,500 to 40,000 nucleotides.

The Frequency of Gap Structure Related to UV Exposure. As reviewed by Takahashi, DNA replication alone should generate some single strand regions in double strand DNA (3). Chromatographic evidence supporting this view was seen recently in studies using the BND-cellulose column for postreplication repair studies (9). Since gap structures in DNA may be generated by replication as well as by the formation of a secondary UV induced lesion, the frequency of occurance and fine structure were determined for DNA preparations without preselection with BND-cellulose. Table I lists the types of molecular species observed and the respective numbers of structures seen after scoring 100-188 fragments selected at random.

DISCUSSION

Table I demonstrated the association between an uncomplicated gap and post UV replication. Joint recombinant molecules were not found in our preparations despite repeated attempts using a different DNA isolation procedure. Recent studies by Takahashi(5) previously discussed in this paper demonstrated UV induction of joint recombinant molecules. Indirect support exists for the association of recombination with postreplica-

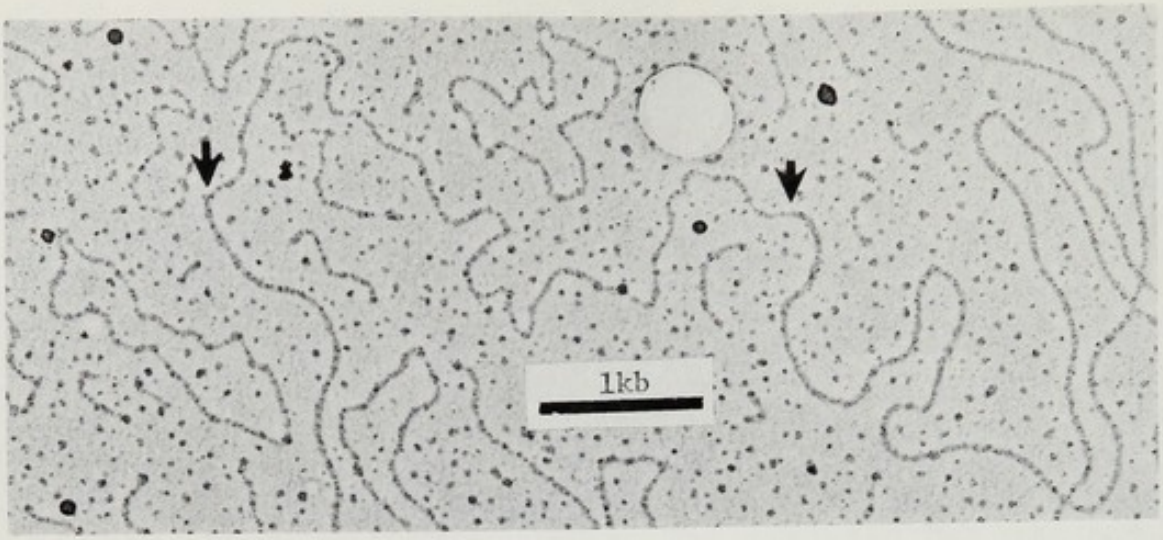

Figure 2. Electron Micrograph of DNA from E. coli prepaired as described previously, except the DNA is spread by the aqueous spreading technique and is not preselected by chromatography.

tion repair as shown by Rupp et al. (10) and Ganesan (11). Recombinant molecules may be more prevalent as the post UV replicated daughter strands age beyond the 15 minutes allowed in this paper. The possibility of joint recombinant molecules originating from UV lesions in non-replicated regions of the DNA is less probable with bacterial DNA than for homologous pairing of phage DNA in multipli-infected cells as seen in the Takahashi system (5). The reason for absence of gaps of less than 800 nucleotides in our observations is unclear.

In this paper BND-cellulose chromatography of DNA fragments from an excision deficient E. coli mutant strain has allowed selection, concentration, and amplification of DNA fragments with single strand regions in double strand DNA. Gene 32 protein stain with the ϕ×174 reference molecules has facilitated visualization and measurement of the proposed post-replication repair daughter strand gap structure. EM studies

TABLE I
FREQUENCY ANALYSIS OF DNA FRAGMENTS CONTAINING SINGLE STRAND REGIONS[a]

	Completely double stranded	Single strand end pieces	Gaps with internal denatured ends	Uncompli-cated gaps	Total counted
No UV	82	16	6	0	100
UV	74	18	8	0	100
UV + replication	115	52	11	10	188

[a]The DNA fragments were not preselected by BND-cellulose chromatography.

with gene 32 protein stain may be useful in mapping the structures and sequence of events from a primary chemical or physical insult to the final repair of DNA.

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A GENERAL METHOD FOR ISOLATION OF REPAIR-DEFICIENT MUTANTS

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ABSTRACT We have developed a rapid method for isolation of UV-sensitive mutants of Escherichia coli. The selection method involves production of growth and/or division delay in repair-deficient cells with low UV fluences, and killing of normally growing repair-proficient cells by penicillin treatment. Using this technique we have achieved about a 3000-fold enrichment for photoreactivationless (phr⁻) mutants of E. coli K-12. We have also shown that the method can be used to isolate mutants deficient in dark repair functions.

INTRODUCTION

Ultraviolet (UV)-sensitive mutants have been an important tool for understanding repair mechanisms in microorganisms. Such mutants have been isolated either by Howard-Flanders and Theriot's "suicidal-phage-reactivation" method (1) or by direct screening of mutagenized cultures (2,3,4,5). Some other mutants isolated for deficiencies in recombination or replication have been found to be UV-sensitive: rec (6), polA (7), lig (8). None of these approaches was practical for isolation of a number of phr mutants needed for genetic analysis of the photoreactivating enzyme in E. coli. Therefore we developed a new method for isolation of phr mutants. This method involves production of growth and/or division delay in repair-deficient cells with low UV fluences (9) and killing of growing repair-proficient cells by penicillin treatment (10,11).

RESULTS

1. Penicillin killing of UV-irradiated and UV-irradiated-photoreactivated cells. E. coli AB1886 (uvrA6) was chosen for isolation of phr mutants because its Uvr⁻ phenotype makes possible a large discrimination in survival and growth between phr⁺ and phr⁻ cells at low UV fluences. This strain does not show postirradiation growth delay in the rich K medium (12) we found necessary for efficient killing by penicillin. Nevertheless the cells do show postirradiation division delay. Since the division septum of growing cells is

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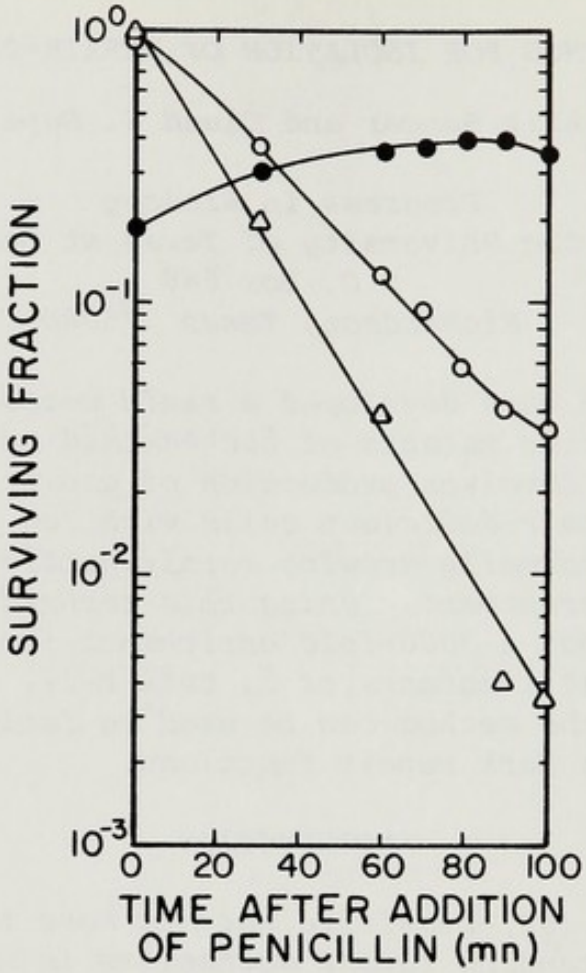

FIGURE 1. Penicillin survival of UV-irradiated AB1886. Cells growing in K medium (12) containing 12% sucrose and 0.01M $MgSO_4$ were irradiated at a density of 10^8 cells/ml and either photoreactivated or kept dark. Growth was continued in the presence of 330 units/ml penicillin, and at the indicated times dilutions made into H_2O at 50°C were plated. Δ Non-irradiated cells, ● UV-irradiated (2.0 J/m^2) and 0 UV-irradiated and photoreactivated cells.

more sensitive to penicillin than the rest of the cell envelope (13), dividing cells are preferentially affected and become sensitive to osmotic shock after treatment with low concentrations of penicillin (14). In the reconstruction experiment shown in Fig. 1 we subjected the cells to osmotic shock after treatment with 330 units/ml of penicillin. As can be seen, the combined UV irradiation-photoreactivation-penicillin-osmotic shock treatment resulted in about a 10-fold enrichment for phr⁻ cells. In other experiments the enrichment factor for phr⁻ cells (simulated by keeping the culture in dark) varied from 10-30 fold. It is also evident that enrichment by about 100-fold can be obtained for uvr⁻ cells. (We use the nonirradiated cells as our uvr⁺ control here because uvr⁺ (AB1157) cells irradiated with the fluence used in this experiment do not show measurable division delay).

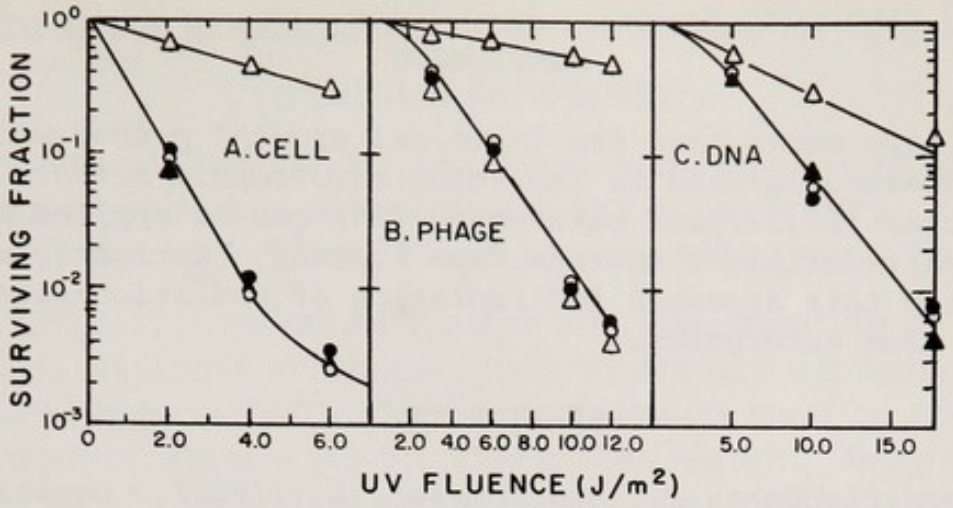

FIGURE 2. Photoreactivation of cells, phage and transforming DNA. AB1886 (uvrA6) and CSR06 (uvrA6 phr-1) cells were either UV-irradiated or infected with UV-irradiated phage, or transformed with UV-irradiated R6K DNA and then exposed to photoreactivating light. Survivals were determined by colony count, plaque count or ampicillin resistant colony count, respectively. (A) Cell survival. (B) T4v1 phage survival and (C) R6K plasmid DNA survival. ● Dark and ○ light survivals of CSR06 or of T4v1 phage and R6K DNA on CSR06. ▲ Dark and △ light survivals of AB1886 or of T4v1 phage and R6K on an AB1886 host.

2. Isolation of phr mutants. An AB1886 culture, mutagenized with nitrosoguanidine, was successively subjected to UV irradiation, photoreactivation, penicillin treatment and osmotic shock. The surviving cells were grown, and the cycle repeated. At each cycle part of the culture was kept in the dark (as a simulated phr⁻ control) and treated similarly. By comparing the survival of the culture kept dark to that of the photoreactivated culture at the end of each cycle, we computed the enrichment obtained for any phr⁻ cells present in the photoreactivated culture. At the end of 5-cycles the total enrichment factor was about 3000. Three out of 77 colonies tested from the cycled culture were phr⁻.

3. Characterization of phr⁻ mutants. The phr⁻ mutants isolated were identified by the qualitative screening assay of Harm (3) and were named CSR06 (phr-1), CSR58 (phr-2) and CSR70 (phr-3). CSR06 was chosen for further studies. Fig. 2 shows the results of different in vivo assays used to test the photoreactivation ability of this strain. These assays showed no detectable PRE activity in CSR06.

CONCLUSION

We have shown that the "suicidal growth" method which has been successfully used in isolating auxotrophic mutants of prokaryotes (10,11) and eukaryotes (15) can be applied to isolate repair-deficient mutants from E. coli. Currently we are evaluating this approach for isolation of radiation-sensitive mutants from eukaryotes.

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HISTORICAL PERSPECTIVES AND KEYNOTES ON DNA REPAIR

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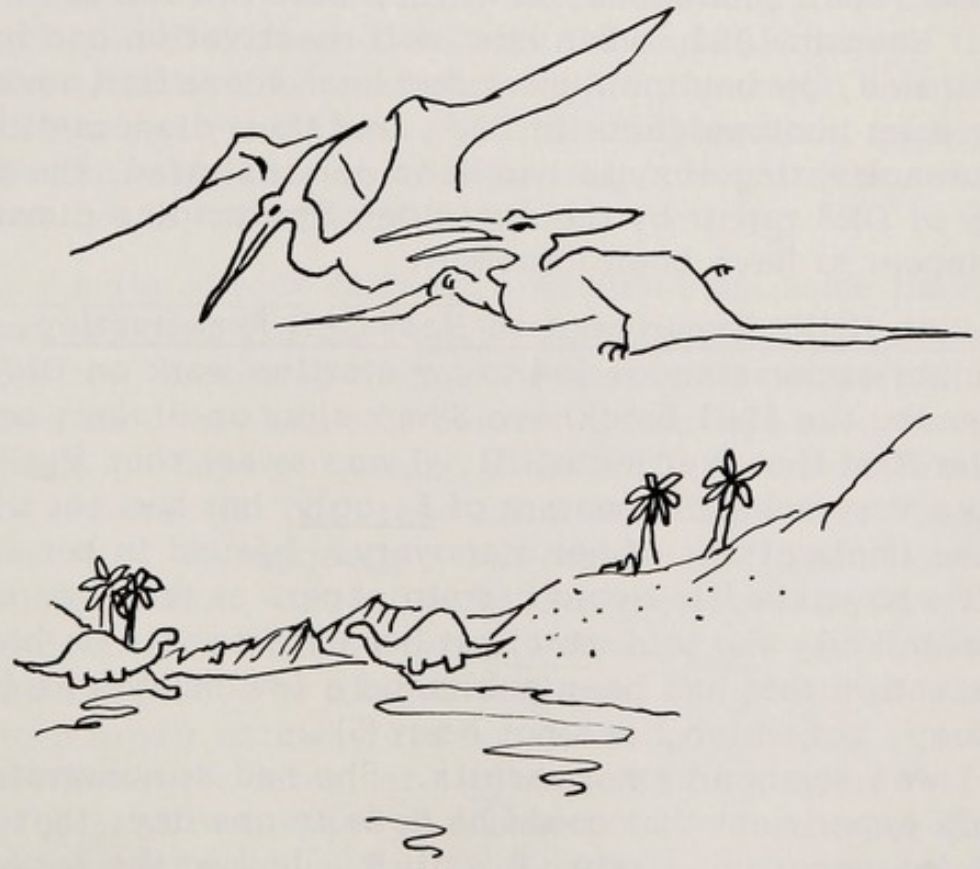
ABSTRACT The identification and investigation of bacterial mutants and human inherited traits with repair defects have led to questions that now must be answered through painstaking biochemical research. Recent work on recA$^+$-dependent processes is opening the way to a fuller understanding of early steps in recombinational repair and the many enzymes required for this remarkable process.

Historical Perspectives. When Phil Hanawalt asked me if I would give the keynote address on historical perspectives in the field of DNA repair, I began to browse through early scientific literature and came across what must be the very first reference to DNA repair.



"Some of these youngsters have come up with a terrific new idea - DNA Repair"

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Looking back to the beginning of my scientific career, I recall that one of the first scientific meetings I attended in London, in 1944, was on Dose Rate Effects in the actions of X-rays on biological systems. In studies on chromosome breaks and lethal effects in the cells of growing bean root tips, X-rays were found to be less effective if delivered in exposures lasting two hours or more, rather than in a few minutes (1,2). Although by hindsight, these observations could have been interpreted in terms of DNA repair, we must remember that at that time, even the role of DNA chromosomes was unknown. At that time, we were amazed that biological systems were so much more sensitive than inanimate objects to the effects of X-rays. Why, therefore, should anyone think about DNA repair? Similar dose rate effects observed in cultured Chinese hamster cells were referred to as Elkind recovery (3), and little effort was made to explore the underlying mechanisms or to get at the biochemistry of these processes.

It is interesting to recall that when the Watson-Crick twin helical structure was proposed, it was seen to provide a structural basis for DNA replication and for specificity in genetic recombination (4). The implication of the twin helical structure for DNA repair processes, however, were not foreseen at the time. Even in 1962, when host cell reactivation had been demonstrated, pyrimidine dimers had been identified among the ultraviolet photoproducts in DNA, and their dissociation by photoreactivating enzyme had been demonstrated, the possibility of DNA repair by the excision of pyrimidine dimers does not appear to have been foreseen.

Ruth Hill's Experiment on Host Cell Reactivation. Several fortunate circumstances led to my starting work on DNA repair. I attended the 1961 Brookhave Symposium on Biology and there for the first time met Ruth Hill. I was aware that she had isolated a very sensitive mutant of *E. coli*, but had not thought out the implications of her discovery. I found in her a kindred spirit who wished to avoid certain papers at that symposium. As we talked, she told me about her experiments on host cell reactivation that had been published a few months earlier in Virology, but which I had not seen (5).

I was amazed by her results. She had demonstrated in a simple experiment that could be done in one day, that the ultraviolet-sensitive strain, *E. coli* B_s, lacked the capacity for reactivating ultraviolet irradiated bacteriophages. The only reasonable explanation for her data was that the mutant B_s lacked enzymes that normally repair ultraviolet damage either in the bacterial or phage DNA. Her experiment was compelling.

I returned to New Haven determined to see if I could repeat this result and isolate repair mutants from *E. coli* K12, a strain more favorable for genetic analysis than *E. coli* B. Starting from a strain that carried about ten genetic markers, we isolated ultraviolet-sensitive mutants and were able to map them to the genetic loci, *uvrA*, *B* and *C*. Jane Setlow educated me about pyrimidine dimers and when Dick Boyce joined my laboratory, we set out to determine the fate of pyrimidine dimers in the DNA of bacteria during DNA repair processes. Chromatography was to me a mysterious thing that other people did, but fortunately Dick Boyce was more resourceful, and together we mastered a makeshift radiochromatogram scanner and were able to identify the photoproducts. We were thus able to determine the fate of pyrimidine dimers in the bacterial DNA. Even at this time, we never guessed that dimers might be removed by the endonucleolytic cleavage of the dimer-containing strand. So far as I know, no one in the laboratories of Setlow or Hanawalt, who were also working on this problem, had foreseen that pyrimidine dimers might be cut out of the DNA in oligonucleotides, rather than being monomerized or in some way repaired *in situ*. It certainly came as a complete surprise to me that the moiety containing excised pyrimidine dimers was an oligonucleotide. Even Max Delbruck, who was the omniscient folk hero of bacterial viruses, and who was familiar with the experiments on host cell reactivation, expressed astonishment and pleasure at the result. None of us achieved an Honors grade for scientific vision.

The Puzzle of recA Mutants. At about the same time, John Clark had isolated the first *recA*$^-$ mutants and brought these to my laboratory, reporting them to be highly UV-sensitive. I visualized that they must be defective in a late step in excision repair, perhaps something to do with the strand joining as this was required for both repair and recombination. Sidney Brenner visited us suggesting that repair might be dependent upon recombination, but I did not like the idea. Even when we isolated double mutants carrying *uvrA* and *recA* and found them to be supersensitive to ultraviolet light, the possibility of an alternative repair pathway did not dawn upon me.

Twelve months later, at a conference on genetic recombination at Lake Arrowhead, I learned of the increased frequency of genetic recombination thought to occur near the ends of T4 phage DNA molecules (6). It was only several months later, that it occurred to me that there might be a *recA*-dependent postreplication repair process, initiated by the free ends at gaps opposite pyrimidine dimers. Fortunately, Dean Rupp

had already mastered the skills needed for lysing and sedimenting labeled bacterial DNA in alkali, and within a few weeks was able to report that after ultraviolet-irradiation, the lengths of newly synthesized DNA was limited to approximately the distance between pyrimidine dimers, and the existence of postreplication gaps was inferred. It took much longer to demonstrate by means of density labels that exchanges could be detected between newly synthesized and preexisting DNA strands after ultraviolet irradiation (7).

Postreplication gaps were soon detected in mammalian cells also (8,9), but evidence as to the underlying mechanism remains confusing even today.

Functions Controlled by recA in E. coli. The recA gene stands out from all other rec$^-$ genes so far described, as the phenotype is both extreme and pleotropic. As many authors have commented, the phenotype includes the following: extreme recombination deficiency; high ultraviolet sensitivity; excessive DNA breakdown; defective control of cell division; lack of ultraviolet mutagenesis and ultraviolet reactivation; and failure to produce normal recA protein (protein X) after ultraviolet or nalidixic acid treatment (10).

The number of papers published on recA-related functions increased sharply in 1973 and 1975, and on recA-biochemistry increased dramatically in 1977. No fewer than four laboratories discovered independently that the recA$^+$ gene product is the same as protein X, which is induced when DNA replication is inhibited. Protein X had been described seven years earlier (11). I note, to my shame, that Dr. Inouye sent me a copy of his manuscript in 1970 and that I failed to see the significance of his work, supposing the effects to be secondary to damage-induced exchanges, which would of course have been recA-dependent.

An important contribution to the field was made by the isolation of a λ transducing phage carrying recA$^+$, which had been used for the enrichment of recA protein in cell extracts and tests on different recA alleles (12).

The Induction of λ Prophage. The development of methods for assaying and purifying λ repressor led to the remarkable discovery that λ prophage induction follows the proteolytic cleavage of repressor. Even more remarkable, the protease that cleaves λ repressor in an ATP-dependent reaction, is none other than the recA$^+$ protein. Moreover, the superinducing allele, recA tif, is several times more active as a protease than the wild type allele (13).

It is paradoxical that efforts to understand the nature and function of the recA gene product have led us to a protease. How then are we to learn about the function of recA protein in genetic recombination?

DNA Substrates for Initiating Recombination. At this point I will briefly summarize recent work in our laboratory which is described in greater detail in papers by Cassuto and Ross in this volume. During postreplication repair in ultraviolet-irradiated bacteria, the postreplication gaps and pyrimidine dimers appear to act as substrates for initiating genetic exchanges between sister duplexes. As one exchange was detected for almost every postreplication gap, we felt that if we could produce a test tube full of DNA containing dimers and postreplication gaps, we would have a good substrate for studying early reactions in DNA repair and genetic recombination. Unfortunately, all efforts to produce this material were disappointing.

Crosslinking agents also are efficient recombinogens. In λ phage-prophage crosses, set up so that the damaged DNA does not undergo replication, crosslinked DNA causes exchanges very efficiently. The induced recombination, however, required $uvrA^+$ and $uvrB^+$, indicating that the damaged DNA had to be incised to become an active substrate. The induced recombination was also dependent upon $recA^+$ (14). The use of crosslinked as compared to damaged phage DNA provided us with a way in which the frequency of recombination between homolgous DNA molecules could be substantially increased.

Cutting in trans as a Step in Damaged-Induced Recombination. As recombination is thought to proceed through the cutting and joining of molecules, we thought that cutting might be detected in undamaged molecules under conditions in which crosslinked DNA might cause extensive recombination. Tests of this kind were carried out in E. coli (λ) infected with ^{32}P-λ phages. Most of the radioactive λ DNA formed into covalent circular molecules under these conditions. When cells containing the circularized λ DNA were superinfected with crosslinked λ phages, it was found that the intact molecules were cut, as expected if recombination was initiated between molecules. This phenomenon of undamaged molecules being cut in response to the presence of damaged homologs is designated, 'cutting in trans.' Cutting in trans is dependent upon $recA^+$ but is not greatly affected by other rec^- mutations. Cutting in trans occurs only between homologous DNA molecules, nonhomologous DNA present at the same time in the

cell remains untouched (15).

Cutting in trans may sometimes be an intermediate step in genetic recombination. It depends upon recA$^+$, requires genetic homology, and that the initiating molecule is damaged in a way known to increase the frequency of exchanges.

Cutting in trans in vitro. In an approach to the enzymology of genetic recombination, a system was set up for testing for cutting in trans in crude extracts of E. coli. We chose to use øX form I DNA rather than λ DNA, because of its lower molecular weight and decreased susceptibility to nonspecific endonucleases. Crosslinked form I øX DNA was readily incised in crude extracts and tested for ability to induce cutting in homologous molecules. The experiments gave irregular or negative results for many months. Fortunately, McEntee provided us with his transducing phage λ recA and when extracts were prepared from thermally induced E. coli (λ recA$^+$), we had more success. With these extracts, which are enriched for recA$^+$ protein, the damaged molecules were incised. The undamaged form I molecules were cut in extracts enriched for recA$^+$ protein but not in extracts from recA$^-$ cells. Cutting in trans was detected between damaged and undamaged form I øX DNA molecules, and also between damaged and undamaged ColE1 form I DNA. However, no cutting in trans was detected with damaged øX DNA and undamaged ColE1 molecules. Thus, as in the experiments on phage λ infected cells, cutting in trans was dependent upon enriched recA$^+$, genetic homology, and suitable damage in the initiating substrate molecules.

These experiments demonstrate the presence of an endonuclease activity in recA$^+$ cells that cuts superhelical DNA in response to interaction with damaged homologous molecules (16).

The role of recA protein in genetic recombination. The results of the experiments just described indicate that the recA gene product is required if the damaged DNA is to cause the undamaged homolog to be cut. It follows that recA$^+$ protein acts either directly or indirectly in one or more of the following steps: (1) unwinding one DNA strand in the damaged molecule (2) the catalysis of homologous pairing in between this strand and the undamaged superhelical DNA (3) the endonucleolytic cleavage of the undamaged molecule in response to homologous pairing. Among the many proteins that may be needed for these complex reactions, we expect to find the recA$^+$ protein, single-strand binding or helix destabilizing proteins, an unwinding enzyme and a homology dependent endonuclease.

Hopefully, the elucidation of these reactions will lead to a deeper understanding of the mechanisms of postreplication repair.

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IVAR JOHANSEN

The tragic and untimely death of Dr. Ivar Johansen on the 12th of May, 1976 came as a great shock to his friends and scientific colleagues. He died after open-heart surgery in Oslo at the age of 42.

Ivar was a friend extraordinary. Those of us who got to know Ivar and his wife, Tonni, during his two years at Yale will never forget their wit, their vivacity and genuine affection for friends.

Ivar Johansen took his Ph. D. at the Norsk Hydro's Institute for Cancer Research. He was a Postdoctoral Fellow in the Radiobiological Laboratories at Yale University, worked at the Norwegian Defence Research Establishment, and in 1972 became Docent (Associate Professor) in the Department of Genetics at the University of Oslo. He attended the 1974 ICN-UCLA Meeting on DNA Repair at Squaw Valley (Molecular Mechanisms in DNA Repair. Eds. P.C.Hanawalt and R.B. Setlow, p. 459, Plenum Press, New York, 1975).

As an instinctive collaborator, Ivar Johansen brought a compelling enthusiasm to his scientific work. He is known for his studies on DNA damage and repair in microorganisms. He showed that DNA single-strand breaks could be detected within 0.1 seconds of a pulsed irradiation. He found that oxygen promoted single-strand breaks by X-rays, whereas organic nitroxyls do not. These sensitizing agents also compete differently with endogenous sulfhydryls in the cell. He investigated the cutting of UV-irradiated phage λ DNA in *E. coli* (λ). The incision of UV-irradiated λ DNA by the *uvrA*-controlled endonuclease was normally followed by very rapid repair, but he devised a system in which this cutting could be detected. These studies have since been extended in several important directions by his former collaborator, Erling Seeberg, and have led to new insights into the roles of the *uvr* genes and gene products.

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ENZYMATIC PHOTOREACTIVATION OF DNA[1]

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ABSTRACT The distinguishing hallmarks of photoreactivation of DNA is the enzyme-mediated, light-dependent monomerization of pyrimidine dimers resulting in repair of the DNA and restoration of its biological integrity. Photoreactivation is differentiated from sensitized dimer monomerization by small molecules by the participation of the protein macromolecule and from dimer monomerization by tryptophan-containing peptides and proteins by their wavelength dependence. The photoreactivating enzyme shows three levels of specificity: first, for the length and kinds of nucleic acid; second, for cis-syn cyclobutyl pyrimidine dimers; and third, for photoreactivating photons of wavelength 300-600 nm. Although all photoreactivating enzymes characterized to date show this specificity in function, current reports indicate possible heterogeniety in structure. Most PR enzymes [E. coli, T. domestica (silverfish), H. sapiens, S. griseus] are composed of a single polypeptide chain with a molecular weight of 35,000-40,000 d. However, the yeast enzyme is reported to be composed of two dissimilar subunits with molecular weights 60,000 and 85,000. In addition to the apoprotein portion photoreactivating enzyme holoenzymes contain a smaller molecular weight, non-protein cofactor which is essential for enzyme activity. In the case of the S. griseus enzyme, Eker reports that the cofactor absorbs light in the spectral region of action of the enzyme, and may thus be the chromophore responsible for absorption of photoreactivating light. For the E. coli, yeast and silverfish enzymes, however, the cofactor absorption is mainly at wavelengths less than 300 nm, and thus cannot mediation photoreactivation absorption in the range of 300-600 nm. In the case of the E. coli enzyme, the photoreactivating

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absorption may well result from a new absorption band in the region 300-500 nm which appears when active enzyme binds to dimer-containing DNA.

The specificity of the photoreactivating enzyme for pyrimidine dimers offers great potential for use as an analytical tool: if potential biological damage is reversible by a true photoenzymatic reaction, pyrimidine dimers were important in production of the biological damage. Correlation of a light-mediated recovery event with enzymatic photoreactivation is essential before such a test can be applied. Three major approaches to such a correlation in a biological system are: 1. isolation and characterization of a photoreactivating enzyme from the cells; 2. characterization of the light-dependence and action spectrum of the recovery event in the cells; and 3. correlation of biological recovery and enzyme action (e.g. enzyme levels and extent of photoreactivation; action spectra). Such correlation of biology and biochemistry will provide powerful approaches for resolving the nature of chemical alteration leading to biological damage in complex biological systems, especially at damage levels too low to be analyzed by usual means.

INTRODUCTION

Pyrimidine dimers in DNA can be monomerized by three distinct methods: direct photolysis, sensitized photodestruction and enzymatic photoreactivation. Distinguishing among these possible mechanisms for a biological photoreactivation event is an important problem in current photobiology. We shall examine the hallmarks of enzymatic photoreactivation which show that it can easily be distinguished from direct or sensitized photolysis. We shall also survey three important areas of current research photoreactivation:

1. identification of the mechanism of cellular photoreactivation.
2. molecular architecture of photoreactivating enzymes (PREs).
3. use of photoreactivation as a molecular probe of biological damage.

MONOMERIZATION OF DIMERS

Direct and Sensitized Photolysis of Dimers. Just as pyrimidine dimers are formed by direct absorption of uv, they can be monomerized by absorption of uv photons (1). Direct photolysis occurs only if the radiation is absorbed by the dimer, i.e. radiation in the range 220-300 nm; it does not occur in the wavelength range 300-600 nm, the region of

biological photoreactivation, simply because the dimner does not absorb light of those wavelengths appreciably. Sensitized dimer photodestruction depends on light absorbed by a sensitizer, a small organic molecule (2), an oligopeptide or tryptophan-containing protein (3). Just as in the case of direct dimer monomerization, sensitized dimer photodestruction proceeds only for wavelengths absorbed by the system (dimer plus sensitizer). Thus, for example, photolysis of dimers by tryptophan-containing proteins occurs only in wavelength regions where tryptophan absorbs. The absorption of tryptophan, which approaches zero near 300 nm, does not extend significantly into the region of principal photoreactivation, 300-600 nm, sensitized photolysis by tryptophan-containing proteins thus cannot account for biological photoreactivation phenomena, which occur at wavelengths greater than 300 nm.

Hallmarks of Enzymatic Photoreactivation. Enzymatic photoreactivation is characterized by its constituents and its action: The first essential ingredient is the PRE protein which can be isolated, analyzed and characterized just like any other protein (4,5). The second critical ingredient is the unique action of the photoreactivating enzyme: it binds to regions of DNA containing cis-syn pyrimidine dimers, the enzyme-DNA complex absorbs photoreactivating light, breaks dimers and yields two pyrimidine monomers; the only action of the enzyme is the monomerization of the dimer (there is no incision into the phosphodiester backbone, no new synthesis, no side reactions of the enzyme to yield new photoproducts) (6). The requirement of the reaction for photoreactivating light provides a further "signature" for use in characterizing the enzyme; the characteristic action spectrum of the enzyme allows its direct comparison with that for biological photoreactivation.

IDENTIFICATION OF THE MECHANISM OF CELLULAR PHOTOREACTIVATION

If we want to determine whether a cell can carry out enzymatic photoreactivation, what experiments would we do? First, we must determine if the cells we wish to examine for biological photoreactivation contain photoreactivating enzyme activity. Since biochemical determination of dimer photolysis is generally faster than biological photoreactivation experiments, we can save time and disappointment by looking for the enzyme before we look for its action. The most easily performed of in vitro dimer photoreactivation experiments involves addition of cellular extract to purified bacterial or bacteriophage DNA labeled with ^{3}H-thymidine and subjected to 254 nm irradiation. The enzyme-DNA complex can then be exposed to broad spectrum photoreactivating light (for

example, a white fluorescent bulb), or kept in the dark. [The dark samples should be handled in subdued light; the use of a red bulb for working light provides a conservative safe lamp until the action spectrum for the reaction is known.] The dimer content of the DNAs can then be determined by hydrolysis, chromatography and counting (7).

Once we have determined that the cells contain a photoreactivating enzyme activity, we can purify the enzyme so that we can characterize it without interference from other components of the extract. It is essential that the photoreactivating activity be followed during the purification, not another property presumed to be associated with the enzyme, such as cofactor absorption. We can now characterize the purified activity by determining

1. if it is associated with a macromolecule
2. if that macromolecule and its associated activity are sensitive to proteolytic enzymes
3. the action spectrum for dimer monomerization
4. if the action of the enzyme results in dimer disappearance and in concomitant appearance of monomer pyrimidines.

The later demonstration is best carried out by examining the action of the enzyme on DNA labeled with ^{3}H-cytosine. The DNA is irradiated to produce dimers, then heated to 60° to deaminate only cytosines with saturated 5,6 bonds (i.e. C-T and C-C dimers deaminate to yield U-T and U-U dimers, but cytosine monomers are unaffected.) Photoreactivation of the U-T and U-U dimers yields radioactive monomer U, which (after hydrolysis and chromatography) appears as a new spot on the chromatograms (8).

If we are now satisfied that the cells contain a true photoreactivating enzyme, we can lok for its cellular action. It is critical here to use the same cells in the biological experiments as were studied biochemically: the photoreactivation literature abounds with examples of differences of PRE activity in cell type of an organism (9), in cells of one type from different individuals of the same species (10) or cells of one type grown under different conditions (11,12). We can test for both cellular photoreactivation of dimers, and for photoreactivation of biological damage. The former test has been impeded by the requirement for incorporation of sufficient levels of radioactive nucleotides for analysis. A new technique, of Achey, Woodhead and Setlow (13), developed from a method of W. F. Studier, obviates the need for radioactivity. Cells are irradiated and photoreactivated as usual, their DNA is extracted, treated with dimer-specific endonuclease from Micrococcus luteus, and electrophoresed on alkaline agarose gels. After neutralization of the gel, the DNA is stained with ethidium bromide and photographed. The

size distribution of the DNAs indicates the dimer content: DNAs with higher dimer contents are cleaved by the endonuclease and the resulting small pieces show greater mobility into the gel; if some of the dimers were photoreactivated, the endonuclease, has fewer sites for nicking and the larger DNA has a decreased mobility on gel. We will also want to determine the effect of photoreactivation on biological damage induced by uv, for example mutation or killing. It is important here to check possible adverse or beneficial effects of photoreactivating light alone, or of such light administered before uv exposure, to determine accurately the true magnitude of photoreactivation.

How can we correlate the cellular effects--whether dimer reversal or reversal of killing or mutation--with the photoreactivating enzyme we have studies from these cells? One major method is that of action spectroscopy: if the photoreactivating enzyme was responsible for the cellular dimer reversal and relief from biological damage, the action spectra for the isolated enzyme should coincide with those for the cellular effects. In addition, if we are so lucky as to find cells with decreased specific activity of the enzyme, the decreased activity of the enzyme should be relfected in lower rates of cellular photoreactivation.

We have now established a number of criteria for use in detecting, and characterizing a photoreactivating enzyme, and for identifying its action in the cell. We shall now examine a case history of such an enzyme from mammalian cells.

A Case History: Mammalian PRE. A mammalian photoreactivating enzyme was first reported in human leukocytes: the activity was shown to cause the disappearance of dimers in DNA in a light-dependent reaction, to be trypsin-sensitive, and to have an apparent molecular weight of about 40,000 (14). Additional studies showed that the enzyme had an action spectrum extending from 300 nm to at least 577 nm, with maximum at about 400 nm, and that its action converted dimers to pyrimidine monomers (15). Photoreactivating enzyme activities have been found not only in human leukocytes, but also in bovine bone marrow, in human and murine cells in culture (5), as well as in canine, feline, and bovine corneal cells (16). If this enzyme is present in the cell, can it act on dimers in cellular DNA? For the case of human fibroblasts cells in culture, several studies have shown that the cells can monomerize pyrimidine dimers in their DNA (17,11,12,15). Action spectra for dimer photoreactivation in human fibroblasts have also been determined: the spectra extend from about 300 nm to at least 577 nm, with a maximum about 400 nm. These spectra agree well with those for dimer monomerization by the human leukocyte PRE in vitro, indicating that the

human PR enzyme mediates cellular dimer photoreactivation (18).

Does the action of this PRE mediate biological recovery? This point is particularly important in view of reports that photosensitizers which can produce dimer reversal not only do not mediate biological photoreactivation but instead cause additional inactivation, presumably through formation of new photoproducts (19). The work of Dr. Helga Harm first indicated that mammalian PREs could restore biological activity to transforming DNA (20). Wagner et al. showed that plaque-forming ability of uv-irradiated herpes simplex virus could be restored by photoreactivation in cultured human fibroblasts (21). Sutherland and Oliver have also reported photoreactivation of DNA synthesis inhibition in human fibroblasts (22).

For the case of human cells, one further question is important: does photoreactivation function in DNA repair in man? It has been suggested that the dimer monomerizing activity observed in cultured human cells might result from a component of the culture medium taken up by the cells. Several lines of evidence argue against such a possibility: First, neither medium nor serum components show any photoreactivating activity (15). Second, photoreactivating enzyme activity has been found in tissues taken directly from various mammals and never exposed to culture media (14,15,16, 20). Third, action spectra for PR by human fibroblasts (which were grown in culture medium) agree closely with those for PR activity by the human leukocyte enzyme, (which was not exposed to culture medium) (18). In addition, van der Leun and Stoop have presented evidence for photoreactivation of erythema in human skin (23). We have thus looked for dimer photoreactivation in intact human leukocytes: immediately after withdrawal of the blood sample, erythrocytes are separated from leukocytes by sedimentation; the leukocytes are washed in phosphate-buffered saline, and exposed to 254 nm radiation, then kept in the dark or exposed to broad spectrum photoreactivating light. Samples are then analyzed by DNA extraction, M. luteus uv-endonuclease treatment, and electrophoresis in alkaline agarose gels. Figure 1 shows that photoreactivation decreases the number of uv-endonuclease-sensitive sites in cellular DNA.

MOLECULAR ARCHITECTURE OF PHOTOREACTIVATING ENZYMES

How are photoreactivating enzymes constructed and how does their structure influence their function? All photoreactivating enzymes known share a common function: the light-dependent photolysis of dimers, leading to repair of the DNA. In all cases which have been examined, their only substrate

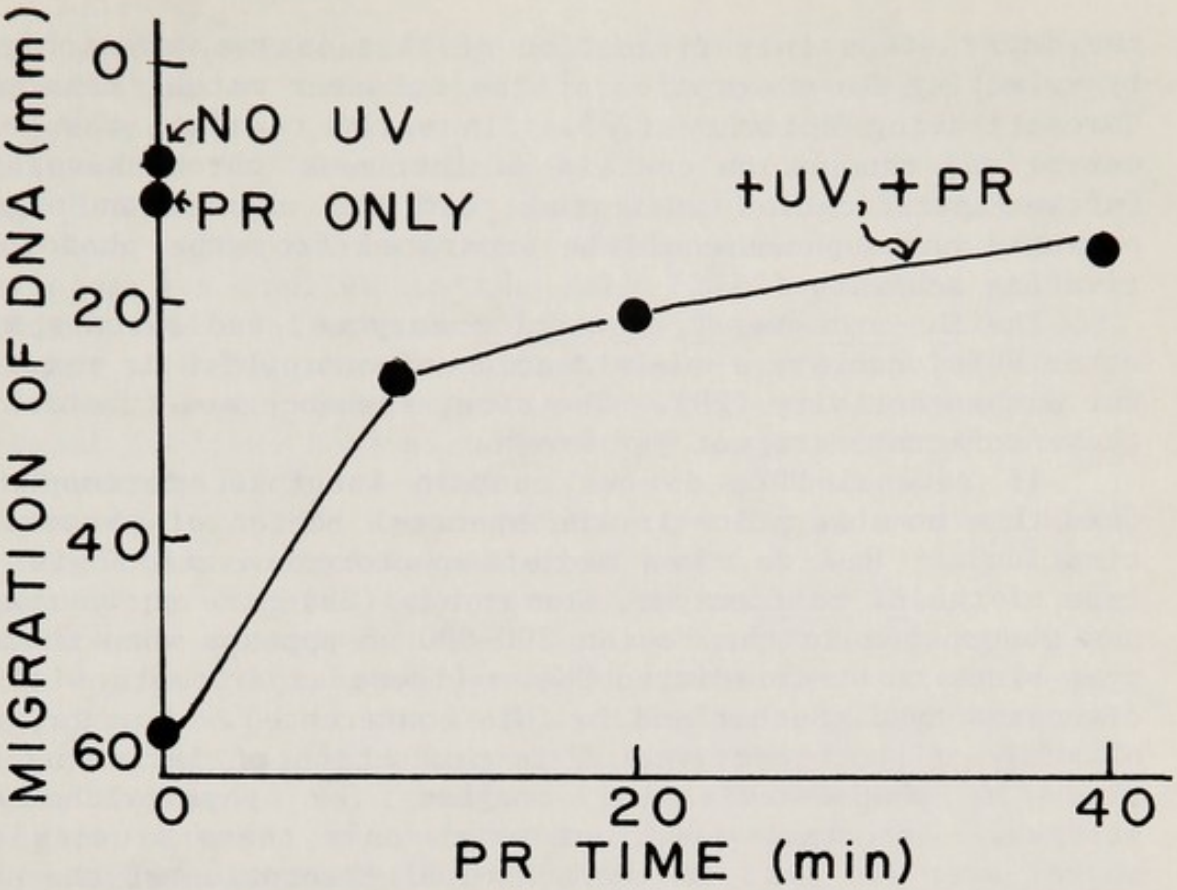

Figure 1. Photoreactivation in human leukocytes.

for the enzyme has been found to be the cyclobutyl pyrimidine dimer in DNA.

Protein Structure. There seem to be at least two different classes of PREs with regards to protein structure: those from Streptomyces griseus, Escherichia coli, Homo sapiens, Thermobia domestica and Cattelya aurantiaca (24), which have apparent monomer molecular weights in the vicinity of 40,000, and those from Anacystis nidulans (93,000) (25) and from yeast, recently reported to consist of two sub-units with molecular weights 60,000 and 85,000 (26). Many PREs aggregate in solution under conditions optimal for enzyme activity, making difficult quantitation of the concentration of active enzymes.

Cofactors and Chromophores. We must distingush here between a cofactor, a small non-protein molecule necessary for activity of the enzyme, but not necessarily involved in light-absorption, and chromophore, a molecule which is involved in absorption of the photoreactivating photon. A signal characteristic of the latter is its absorption in the spectral region of cellular photoreactivation. The only PRE to contain a moiety which meets this criterion is that from S. griseus, although some reservation is necessary here as

the later steps in purification of this enzyme were achieved by selecting for absorption of the cofactor rather than photoreactivating activity (27). In early reports, the yeast enzyme was thought to contain an intrinsic chromophore, but further purification indicated that the absorption of the presumed chromophore could be separated from the photoreactivating activity (26).

The E. coli and T. domestica enzymes, and perhaps some other PREs, contain a nucleic acid cofactor which is required for enzyme activity (28). The size, sequence and function of these cofactors are not yet known.

If several PREs do not contain intrinsic chromophores (and thus no absorption in the spectral region of photoreactivation). How do they mediate photoreactivation? In the case of the E. coli enzyme, Wun et al. (29) have shown that a new absorption in the region 300-500 nm appears when the enzyme binds to uv-irradiated DNA. (These experiments will be discussed by J. Sutherland in this conference). A major goal of study of photoreactivation is production of large quantities of photoreactivating enzymes for physicalchemical studies. Important questions which only these studies can answer are: (1) what is the source of absorption of the photoreactivating photon in other species? (2) do the absorption spectra of other PR enzyme-uv'd DNA complexes reflect the action spectra of photoreactivation in the cells?

PHOTOREACTIVATION AS A MOLECULAR PROBE

The specificity of the photoreactivating enzyme for pyrimidine dimers allows its use as a probe of the chemical damage leading to biological damage. In bacteria and in simple eucaryotes a "photoreactivation test" has been used to evaluate the role of dimers in killing and in mutagenesis (30). The underlying logic of this test is as follows: the photoreactivating enzyme is specific for pyrimidine dimes; thus, if uv-induced biological damage can be prevented by a true photoenzymatic reaction, pyrimidine dimers were important in the production of that damage. Setlow, Hart and their associates have used such a photoreactivation test to provide evidence that dimers are an important lesion in tumorigenesis in the fish Poecilia formosa (31).

The application of such a test to mammalian system has three requirements: (1) a demonstration that the mammalian enzyme is specific for pyrimidine dimers, (2) development of a good system for evaluating uv-tumorigenesis in mammalian cells, and (3) evaluation of any light-induced reaction as photoreactivation or some other light-dependent phenomenon. Evidence is accumulating that human enzyme is specific for pyrimidine dimers and does not act on other DNA photopro-

ducts, even those containing cyclobutyl rings (32). Systems for evaluating uv tumorigenesis are being developed in several laboratories: Chan and Little reported the induction of transformation of mouse cells by uv irradiation (33), although Heidelberger and his associates, using the same cell-line, have not obtained uv transformation unless a co-carcinogen was applied to the cell (34). DiPaulo has reported induction of uv transformation in hamster cells (35). McCloskey and Milo have reported preliminary results of selection of density-independent growth of human cells after uv treatment (36); we have also developed a system for observing transformation of human cells after uv exposure (32).

The use of these systems allows the direct evaluation of the target for uv-transformation in human cells: action spectroscopy can be used to determine if nucleic acids or proteins are the principal target for uv in producing transformation by uv. If photoreversal of transformation can be demonstrated, and if it can be identified as a true photoenzymatic reaction, it will be possible to identify the chemical lesion leading to uv transformation. Preliminary evidence indicates that DNA is the target, and that the uv induction of transformation is photoreactivable.

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WORKSHOP SUMMARY : MECHANISM AND DIVERSITY OF PHOTOREACTIVATION

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The specificity of photoreactivating enzymes for pyrimidine photodimers makes them a very useful tool to elucidate the effect of these chemical changes on the biological behaviour of prokaryotic and eukaryotic systems which have been submitted to the action of UV light. However several points remain to be clarified if one wants to make use of this repair system analytically without any ambiguity as to the interpretation of the results. Several topics have been discussed during the workshop on photoreactivation :

1) the mechanism(s) of action of photoreactivating enzymes
2) the production of photoreactivating enzymes in larger quantities
3) the specificity of photoreactivating enzymes
4) the relationships between photoreactivation and photosensitized splitting of pyrimidine dimers by tryptophan-containing peptides and proteins.

Mechanisms of action of photoreactivating enzymes

The diversity of the structures and mechanisms of action of photoreactivating enzymes remains an intringuing problem. One gets the feeling that every prokaryotic or eukaryotic system has developed its own strategy for the photoreactivation of pyrimidine dimers. Photoreactivating enzymes from many sources have been described (1, 2). Three of them have been the subject of extensive studies at the molecular level, namely, those of baker's yeast, Escherichia Coli and Stretpomyces griseus. Since all photoreactivating macromolecular systems purified until now are proteins or glycoproteins they require a chromophore whose absorption spectrum would correspond to the action spectrum which may extend to 600 nm depending on the system. In addition a cofactor might be required for activity which is not involved in light absorption.

From what is presently known it appears that not only

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the protein varies from one species to another but also the cofactor itself is different. Moreover only one of the purified photoreactivating enzymes, that from S. griseus, described by A. P. M. Eker during this workshop, appears to contain a chromophore whose absorption spectrum is identical to the action spectrum for photoreactivation (3).

Some years ago, the photoreactivating enzyme from baker's yeast appeared to contain a chromophore whose absorption spectrum was similar to the action spectrum. However further purification led to a loss of this absorbing species without loss of the photoreactivating activity (4). Several small molecules, some of which might be oligopeptides, appear to stimulate the activity of this enzyme (H. Werbin, this workshop).

The purified photoreactivating enzyme from E. coli lacks absorption above 300 nm as does the yeast enzyme. How then can we explain photoreactivation in the wavelength range 300-500 nm ?

One possibility could be that the absorption responsible for photoreactivation appears only when the enzyme is bound to UV irradiated DNA. This is what has been demonstrated in the case of the E. coli enzyme by J. Sutherland and his collaborators (5). A new absorption spectrum develops (more easily seen by difference absorption spectroscopy) when the photoreactivating enzyme binds to UV irradiated DNA. It corresponds to the expected action spectrum and disappears after irradiation with photoreactivating light which dissociates the complex.

Whether this result may be extrapolated to photoreactivating enzymes from other sources must await further studies. But it seems likely that photoreactivating enzymes with no absorption above 300 nm (when they are free in solution) but which nevertheless photoreactivate DNA must give rise to an induced absorption when bound to UV irradiated DNA. The origin of this new absorption is not yet clear. J. Sutherland has proposed that the carbonyls at the 2 positions of pyrimidine dimers could provide recognition sites which would participate in either charge transfer or proton transfer reactions. However interaction of some enzyme component with neighboring bases seems to be involved since the action spectrum depends on the DNA base sequence (2). Charge transfer bands due to interaction of aromatic chromo-

phores (including tryptophan) with nucleic acid bases have already been described (6-8). Further studies are required to determine which component of the photoreactivating system is involved in the interaction with nucleic acid bases and pyrimidine dimers.

Cloning of the phr gene

In order to open the way to important studies at the molecular level on the structure and function of the genes involved in DNA repair processes attempts are being made to incorporate these genes into plasmids. During the workshop on photoreactivation, C.S. Rupert and A. Sancar reported that they have succeeded in inserting the gene phr for photoreactivating enzyme of *E. coli* into plasmid pMB9 by *in vitro* recombination.

These studies led to a reevaluation of the location of the *phr* gene on the *E. coli* map which was found to lie counterclockwise from the site between *gal* and *att* λ where it was previously reported to be (A similar conclusion was reached independently by D. Youngs and K.C. Smith).

The number of photoreactivating enzyme molecules per cell has been increased by a factor of 10 as a result of the increase in the number of gene copies. Reduction of the plasmid DNA size as well as an increase in the number of gene copies per plasmid (and per cell) are the next objectives of these studies. It should be noted that pur A^- strains overproduce the photoreactivating enzyme. With rec A^- pur A^- strains containing the pMB9 plasmid carrying the *phr* gene the number of photoreactivating enzymes per cell reaches 1300.

Selectivity of photoreactivating enzymes

For all people who use photoreactivation as an analytical tool to elucidate the biological role of pyrimidine dimers a crucial point is to be sure that only pyrimidine dimers are removed. A recent study by W. Harm, reported at this workshop, raises some doubts concerning this absolute specificity of photoreactivating enzymes and suggests that damages other than pyrimidine dimers might be removed by photoreactivation, especially when irradiation is carried out at long wavelengths (365 nm). Comparing *E. coli* strains which are proficient or deficient in dark repair and at the same time phr^- or phr^+, which have been irradiated at 254 nm or 365 nm,

W. Harm's experiments suggest the formation of photoenzymatically repairable photoproducts (10-20 % of total damages) which are not dark repaired after 365 nm irradiation. These photoproducts are absent or rare after 254 nm irradiation. Similar results were obtained after exposure of cells to sunlight ($\lambda > 360$ nm). There remains several questions to be answered before a definite conclusion can be reached (protection of DNA against certain types of damages upon 365 nm irradiation in the phr^+ strains rather than photoreactivation of these damages, analysis of these effects at different irradiation wavelengths). If the present experiments were to be interpreted as a result of photoreactivation of non-dimer type damage, this would obviously require a reevaluation of some experiments and a more careful analysis of the photorepair processes. An identification of the chemical nature of the photorepairable non-dimer damage will be required.

Relationships between photoreactivation and photosensitized splitting of pyrimidine dimers

Pyrimidine dimers can be monomerized by the direct absorption of short wavelength UV radiation (9). As a matter of fact the percentage of thymines converted into dimers in native DNA is the result of a photoequilibrium between the formation and splitting of dimers which depends on the excitation wavelength. Dimers do not absorb above 300 nm and cannot be monomerized at wavelengths longer than 300 nm.

The splitting of pyrimidine dimers can be photosensitized by different types of molecules (see reference 10 for a review). Indole derivatives are one class which act as photosensitizers. More recently we showed that oligopeptides and proteins containing tryptophan residues could also act as photosensitizers. For example the protein which is coded for by gene 32 of phage T4 binds to UV irradiated DNA and photosensitizes the monomerization of thymine dimers. However this reaction differs from that of photoreactivating enzymes in several respects (11). The action spectrum for the reaction photosensitized by tryptophan-containing molecules does not extend to wavelengths where photoreactivating enzymes have their maximum activity. Even if the charge transfer absorption arising from stacking interactions between tryptophan and nucleic acid bases is considered (7, 8, 12) it is not possible to account either for the high efficiency of photo-

reactivating enzymes in the wavelength range 350-450 nm or for the maximum wavelength of the photoreactivation action spectrum (2). Moreover it has not been demonstrated yet that photoinduced monomerization of thymine dimers is the only reaction taking place in complexes formed by UV irradiated DNA with gene 32 protein. Photoreactivation not only means photosensitized splitting of pyrimidine dimers but also restoration of the original biological properties of photoreactivated DNA.

There are probably many molecules present in cells which could mediate the photosensitized splitting of pyrimidine dimers in DNA and it would be worthwhile to look for such non-protein photoreactivating systems. They might provide a new and easier way of eliminating pyrimidine dimers in situ. However it would be necessary to demonstrate that such molecules do not simultaneously induce the formation of more harmful damage into DNA or in other cell components.

Tryptophan-containing proteins which bind to DNA might play a role in the photosensitivity of DNA in cells exposed to UV light. Tryptophan stacked with bases has been shown to deactivate both the singlet and triplet excited states of nucleic acid bases and should therefore prevent dimer formation (8, 13). This would also be true for tyrosine stacked with pyrimidine bases (14). Any aromatic amino acid inserted between two pyrimidines would also prevent dimer formation for steric reasons. Further work has to be carried out before one can conclude that DNA-binding proteins are involved either in protecting DNA from UV radiations or in photoreactivating DNA containing pyrimidine dimers.

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SOME PROPERTIES OF A DNA PHOTOREACTIVATING ENZYME FROM *STREPTOMYCES GRISEUS*

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ABSTRACT Photoreactivating enzyme from *Streptomyces griseus*, molecular weight 43000, appears to consist of a single protein chain. An optimal pH of 7.0, an optimal ionic strength of 0.04 and an isoelectric point at 4.7 was found. Highly purified enzyme preparations exhibited an absorption spectrum in the visible. The presence of an intrinsic chromophore is discussed.

INTRODUCTION

As early as 1949 Kelner (1) described photoreactivation in *S.griseus*, but it lasted till 1970 before the *in vivo* photoreactivation in this microorganism was studied more extensively by Jagger *et al* (2). A few years ago we mentioned the isolation of a factor from vegetative *S.griseus* cells showing photoreactivating activity *in vitro* (3). This factor achieved splitting of pyrimidine dimers in UV-irradiated DNA into the constituent pyrimidine monomers using blue light as a physical cofactor. Since inactivation by heat and proteases demonstrated its proteineous character, it was concluded that we were dealing with a true photoreactivating enzyme (PRE).

METHODS

Highly purified PRE from *S.griseus* was obtained as described earlier (3). PRE activity was measured with the *Haemophilus influenzae* transformation assay (4). Absorption spectra were recorded with a Beckman UV 5260 spectrophotometer and corrected luminescence spectra were measured with a spectrofluoriphosphorimeter constructed in our laboratory. The provisional action spectrum was measured with the excitation part of the spectrofluoriphosphorimeter.

RESULTS AND DISCUSSION

The pH-dependence of photoreactivating activity is shown in fig. 1. The optimum lies around pH 7.0 and no activity was found below pH 4 or above pH 9.5: in both cases rapid irreversible inactivation took place.

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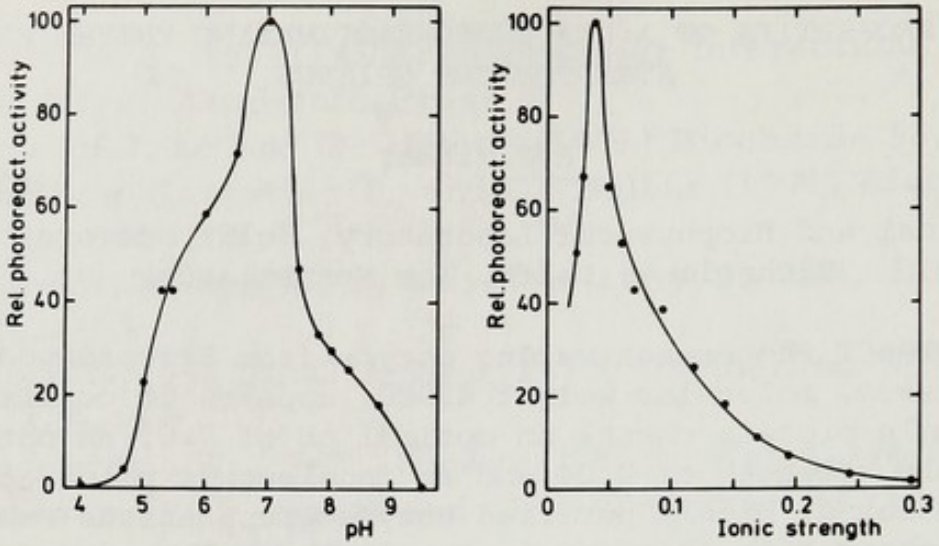

FIGURE 1. (left panel) Influence of pH on photoreactivating activity.

FIGURE 2. (right panel) Influence of ionic strength on photoreactivating activity, measured by variation of the NaCl-concentration in 0.01 M K-phosphate buffer pH 7.0.

The influence of the ionic strength is shown in fig. 2. A maximum was found at I = 0.04.

The molecular weight of PRE from *S.griseus* was estimated by gelchromatography to be 43000. SDS-polyacrylamide gel-electrophoresis revealed a single band, molecular weight 49000, indicating that our PRE consists of a single protein chain. An isoelectric point of 4.7 was found by isoelectric focussing in a glycerolgradient.

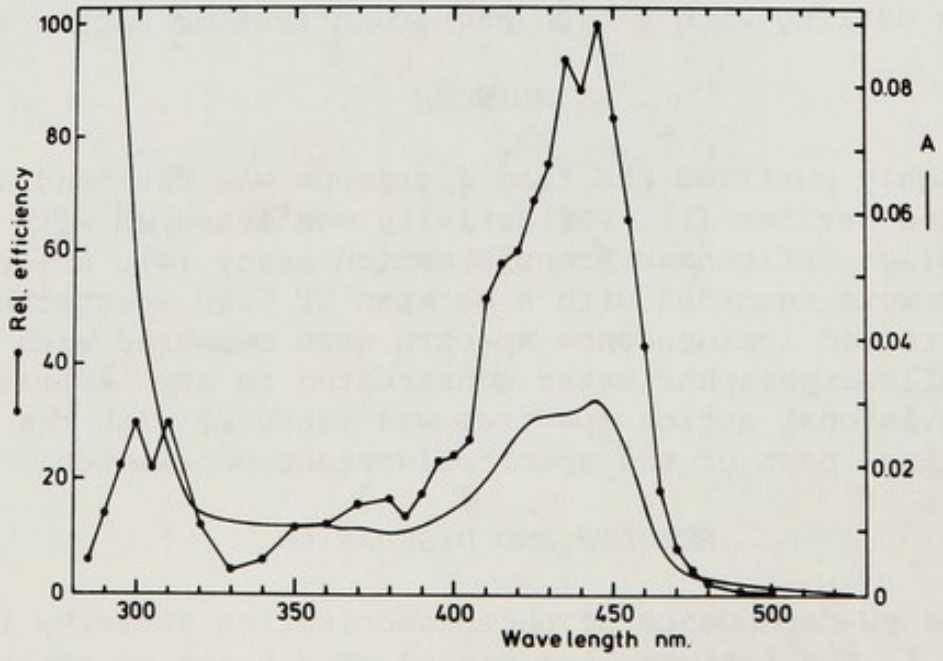

FIGURE 3. Comparison of the absorption spectrum of native PRE (——) and the provisional *in vitro* action spectrum (●——●).

All these properties are not very different from those found for PRE's from other sources. However, PRE from *S.griseus* exhibits an interesting absorption spectrum in the visible region, with a maximum at 445 and a shoulder at 425 nm, coinciding very well with its action spectrum (fig. 3). In addition to the visible region extensive photoreactivation below 320 nm was found, although partially masked by the rapid inactivation of transforming DNA at these wavelengths.

The absorptionband suggests the presence of an intrinsic chromophore. Therefore the fluorescence properties of the enzyme were studied. Freshly prepared native PRE showed a low fluorescence emission, which was considerably enhanced by denaturation, see fig. 4. Of course this could be the result of coinciding denaturation of a contaminating protein. When native PRE was kept at 10 ^{0}C a slow decrease of biological activity took place accompanied by an increase of fluorescence intensity. This increase was much smaller after addition of DNA and with UV-DNA no increase at all was observed, indicating that the species giving rise to a fluorescent product was stabilized by UV-DNA, the substrate for PRE.

In order to achieve heat denaturation after gel-chromatography a coil at 65 ^{0}C was applied between column and flowfluorimeter. It was shown (fig. 5) that denaturation of PRE is accompanied by the release of a low molecular highly fluorescent compound, likely to be an intrinsic chromophore.

With the same experimental set-up co-chromatography

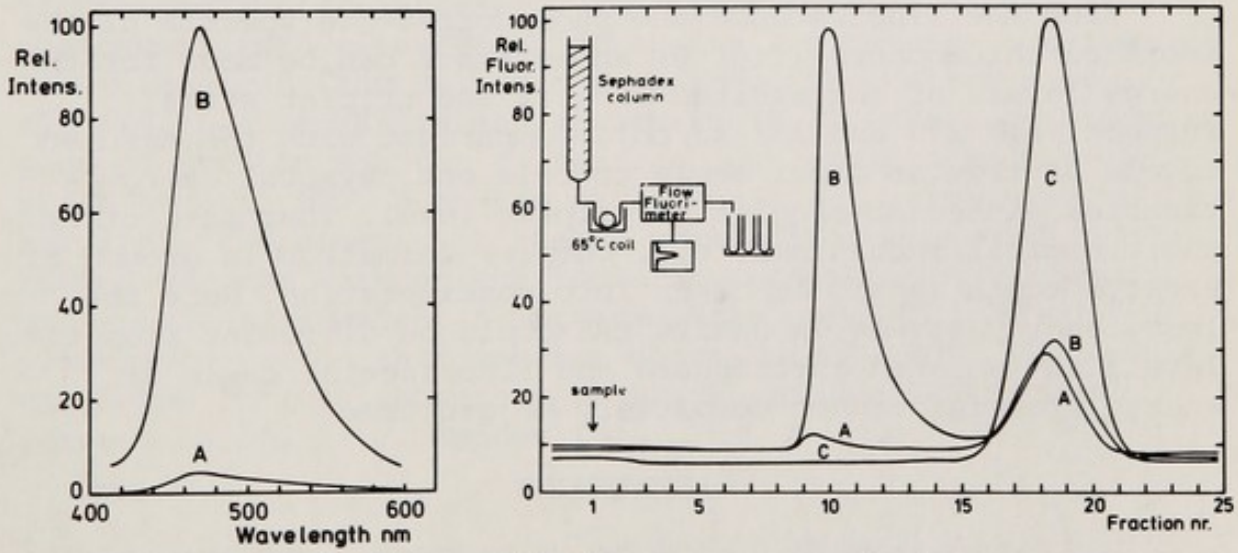

FIGURE 4. (left panel) Fluorescence emission spectrum of native PRE (curve A) and heat denaturated PRE (curve B), λ exc. 400 nm.

FIGURE 5. (right panel) Elution profiles of a PRE/ chromophore mixture on a Sephadex G - 50 (1 x 44 cm) column. Curve A: coil temp. 10 ^{0}C. Curve B: coil temp. 65 ^{0}C. Curve C: sample denaturated before application to column.

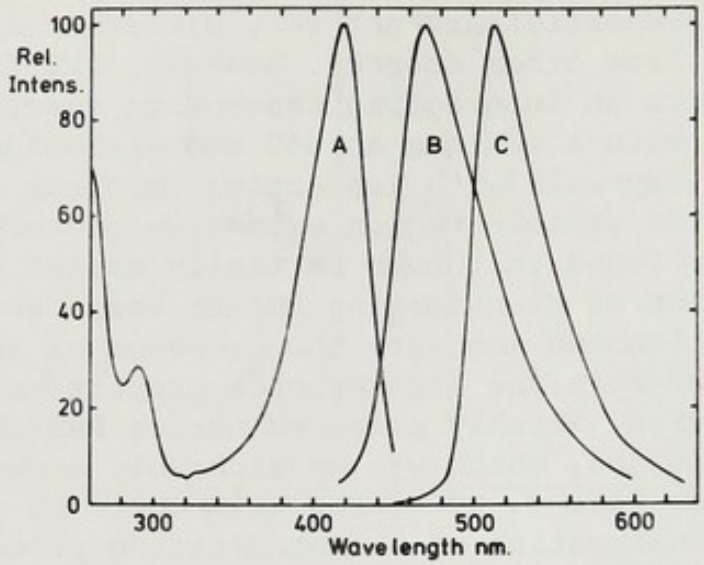

FIGURE 6. Luminescence spectra of isolated chromophore.
A: fluorescence excitation spectrum, λ emission 480 nm.
B: fluorescence emission spectrum, λ excitation 405 nm.
C: phosphorescence emission spectrum, λ excitation 423 nm.

of biological activity and chromophore fluorescence was found in 5 different chromatographic systems.

No absolute proof for the existence of a chromophore in our PRE can be given, since experiments to achieve reconstitution between isolated protein part and isolated chromophore were not successful in our hands. Yet, in view of the results mentioned above we consider the presence of such a chromophore as highly probable.

From the fluorescence and phosphorescence spectra of the isolated chromophore (fig. 6) an estimate can be made for the energy levels of the excited singlet and triplet state, respectively 270 and 252 kJ/mol. Comparison with the assumed levels of thymine dimer seems to rule out physical energy transfer either at singlet or triplet level. Therefore other photochemical mechanisms, e.g. complex formation in ground or excited state should be taken into consideration. None the less, energy levels in native PRE could be different from the levels of isolated chromophore and also special cases of energy transfer cannot completely be excluded.

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LOW MOLECULAR WEIGHT SUBSTANCES THAT ENHANCE DNA PHOTOLYASE ACTIVITY[1]

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ABSTRACT A low molecular weight substance that enhances the activity of DNA photolyase purified by affinity chromatography has been isolated from acidified autolyzates of Baker's yeast. The substance, purified by ion-exchange and gel filtration chromatography, reacts positively with ninhydrin and absorbs at 248-250 nm. This differs from its excitation maxima at 280 nm and 358 nm, leaving unresolved whether the fluorescence of the activator is that of a covalently or non-covalently bound chromophore.

INTRODUCTION

Recently Mortelmans and coworkers (1) reported that photoreactivation in mammalian cells was demonstrable only for cells that had been grown in Dulbecco's medium. They attributed photoreactivation to either DNA photolyase or photosensitized cleavage of dimers by a substance acquired or biosynthesized from Dulbecco's medium. They cited some of the findings of Hélène and coworkers (2,3), for example, that gene 32 protein and the tripeptide lysyl-tryptophyl-lysine, which bind to DNA, both can photosensitize cleavage of cyclobutyl dipyrimidines. It seemed worthwhile, therefore, to review our own work on the isolation and purification of small molecular weight substances from acidified Baker's yeast autolyzates that enhance photoreactivation. This work was begun in our laboratory by Dr. S. Minato (4) and was pursued by Dr. J. J. Madden (5).

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METHODS

Initially (4), substances that increase the activity of photolyase were obtained by (a) acidifying a toluene autolyzate of yeast to pH 3.0; (b) stirring 3 h at 37°C; (c) centrifuging; (d) neutralizing the supernatant and freeze-drying it. The crude material enhanced photolyase activity when the assay was performed in buffers containing either 2-mercaptoethanol or glutathione. In fact the enhancement depended upon the extent of photolyase purification (TABLE I), indicating either a progressive loss of some substance required for photoreactivation or the progressive inactivation of some essential group on the enzyme.

The substances that enhance photolyase activity were called activators and although isolatable by paper chromatography and paper electrophoresis (4), they were unstable. We finally purified an activator by the procedures outlined in Scheme 1 followed by gel filtration of CAYE-III-I on Sephadex G-25 twice (5). Success depended upon working in yellow light.

RESULTS AND DISCUSSION

The purified activator ran as a single spot in two solvent systems and reacted positively with ninhydrin. It had an estimated molecular weight of 450 daltons and in neutral and acid solutions absorbed maximally at 248-250 nm. Excitation maxima were observed at 280 nm and 358 nm when emission was

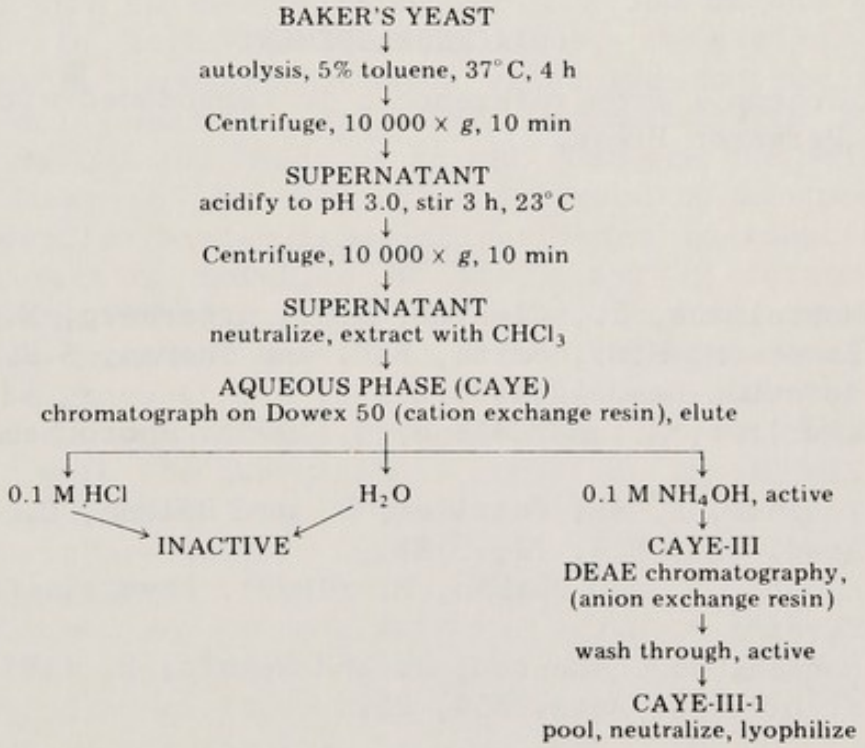
TABLE I

ENHANCEMENT OF PHOTOLYASE ACTIVITY AT SEVERAL STAGES OF ITS PURIFICATION

Purification Step	Ratio of Specific Activities Activator Present/Activator Absent	
	A(mercaptoethanol)	B(glutathione)
Autolyzate	0.99	0.94
$(NH_4)SO_4$(35-55%)	1.03	1.4
Phosphocellulose chromatography	1.72	1.6
Affinity chromatography	1.74	2.4

SCHEME I

PARTIAL PURIFICATION OF ACTIVATOR FROM BAKER'S YEAST [5].



measured at 440 nm. When excited at 290 nm, the activator emitted at 350 nm and 440 nm. Fluorescence at 440 nm was dependent upon both the excitation wavelength and the pH (5). Surprisingly, while both the activator and photolyase fluoresce, their excitation spectra do not reflect their absorption maxima, suggesting that the enzyme's near UV action spectrum maximum is a function of the dimer-enzyme complex. Significantly, near UV absorption can be induced in the activator by lowering the dielectric constant of the medium, and possibly a similar phenomenon could occur upon the binding of the dimer to the enzyme yielding an absorption maximum for the complex more in keeping with its action spectrum. It must, however, be remembered that activator can be isolated from PhR^+ and PhR^- yeast cells, and its role in photoreactivation *in vivo* remains unclear.

ACKNOWLEDGMENT

Scheme 1 from reference 5 is reproduced with permission from Pergamon Press.

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MECHANISM OF ACTION OF THE PHOTOREACTIVATING ENZYME FROM E. COLI: RECENT RESULTS.*

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ABSTRACT Purified photoreactivating enzyme (PRE) from E. coli has an absorption maximum at 257 nm, but no well defined absorption band for $\lambda > 300$ nm, the wavelength region which is required for photorectivation. When PRE is mixed with ultraviolet irradiated DNA (uvDNA), there is an increase in absorption for $\lambda > 300$ nm. Absorbance changes are not observed if PRE is mixed with unirradiated DNA nor is the increase for $\lambda > 300$ nm due to an increase in light scattering. Thus, the long sought "chromophore" appears to be a molecular complex which exists only when E. coli PRE interacts with dimer-containing DNA and leads us to ask what are the moieties of the dimer and the PRE which interact to produce the new absorption band. A preliminary step in identifying the interacting moieties of the dimer is determining whether the PRE approaches the dimer from the major or the minor groove of the DNA. If the PRE approaches from the major grove it can interact with the cyclobutyl ring while if it approaches from the minor groove, it might interact with the two carbonyl groups at the 2 positions of the pyrimidine ring which are common features of all photoreactable dimers. Recent data show that the basic peptide antibiotic netropsin, which is known to bind in the minor groove, inhibits binding of the PRE to uvDNA. This result supports the hypothesis (but does not prove) that the carbonyl groups at position 2 of the pyrimidine rings may be key moieties in the interaction between the E. coli PRE and uvDNA.

Photoreactivating enzyme (PRE) from E. coli, purified by several different methods, has an absorption maximum near 257 nm, but no well defined absorption band at wavelengths longer than 300 nm--i.e. in the spectral region where absorption is required for enzymatic activity (1,2). The 257 nm peak is due to RNA which copurifies with the PRE and which may be required for activity. Partial enzymatic digestion of the RNA greatly reduces the magnitude of the uv absorption and

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permits the spectroscopic determination of tyrosine by the method of Edelhoch (3), without revealing any indication of a well defined absorption band for wavelengths greater than 300 nm (Snapka, this meeting). In contrast, PRE from S. griseus shows an absorption spectrum similar to its action spectrum (4). Thus there appear to be at least two classes of photoreactivating enzymes with different mechanisms of action.

Wun et al. (2) showed that new absorption appears between 300 and 450 nm when E. coli PRE interacts with ultraviolet irradiated DNA but not with unirradiated DNA. This absorption disappeared when irradiated with actinic intensities of radiation in the range 313 to 436 nm. The action spectrum for disappearance of the induced absorption band is similar to both the shape of the new absorption and the action spectrum for dimer monomerization by the E. coli PRE (5). Thus, it appears that the "chromophore" responsible for the absorption of PR light is not a distinct molecular species or moiety but rather a complex formed when E. coli PRE interacts with uvDNA. Both charge- and proton-transfer complexes have properties which could account for the appearance of the new absorption.

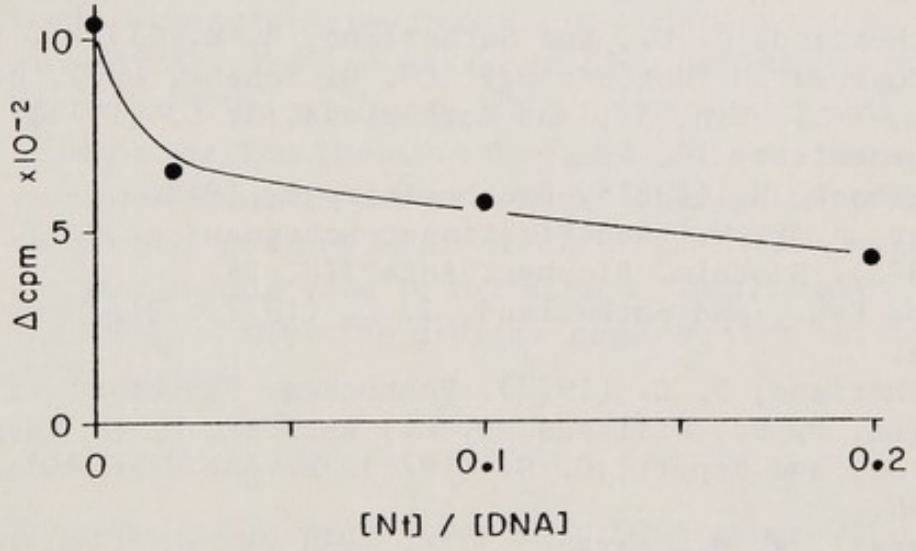
While we will assume that the absorbing complex involves the interaction of some moiety of the enzyme with some part of a dimer, other possibilities cannot be excluded at present. In addition to the interaction which produces the chromophore, there must be interactions involving other regions of the DNA since the PRE will not act on isolated dimers.

A major step in determining the mechanism of action of the E. coli PRE is identification of the moieties which interact to form the chromophoric complex. Sutherland (6) pointed out that the ability of PRE to act on dimers containing thymine, cytosine and uracil suggests that the PRE interacts with either the carbonyl groups at the 2 positions of the pyrimidine rings or at the 6 positions. The 6 positions are directly involved in the cyclobutyl ring while the carbonyls at the 2 positions would provide convenient recognition sites via hydrogen bond formation and are likely candidates to participate in either charge transfer or proton transfer reactions.

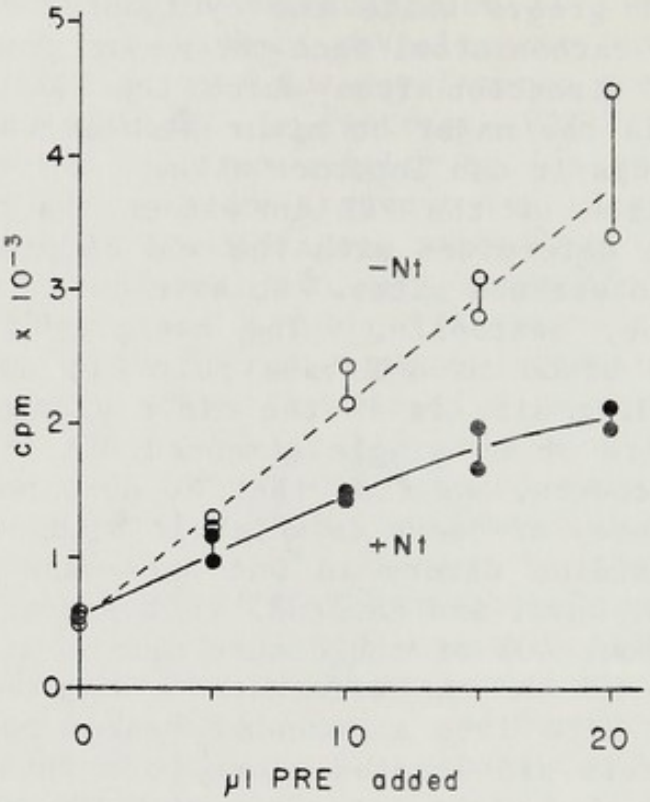
Fortunately, the geometry of the DNA helix provides a method of distinguishing between interaction of the PRE at the 2 position as opposed to the 6 position. Formation of a dimer causes a disruption in the double helix. However, the disrupted region is quite small, the best estimate being only four base pairs including the two base pairs involved in the dimer (7). This distorted region is sufficiently small that the major and minor grooves of the DNA retain their identities at a dimer site. The 2 position carbonyl groups project

into the minor groove while the cyclobutyl ring along with the 6 position carbon atoms face the major groove. Thus determining the direction from which the PRE approaches the dimer (i.e. via the major or minor grooves) also determines with which groups it can interact with.

The location of the PRE in either the major or minor grooves can be determined with the aid of probes which bind to DNA at predetermined sites. We have data on the effect of one such probe, netropsin. The basic peptide antibiotic netropsin (Nt) binds to A-T base pairs in double stranded, DNA. The binding site is in the minor groove. Nt does not bind to GC pairs or to single stranded DNA (8,9). Contrary to previous reports, we find that Nt does not greatly perturbe the geometry of bases to which it binds (10). The presence of pyrimidine dimers in DNA decreases the binding of Nt (Sutherland, Duval and Farland, in preparation). In calf thymus DNA, about 40% of the dimers eliminate a Nt site completely while 60% do not eliminate a site but may convert a very strong site into a somewhat weaker one. Thus about 60% of the dimers are located close to a functional Nt site which when occupied might interfere with the binding or activity of a PRE. If saturating concentrations of Nt are added to uv irradiated DNA prior to the addition of E. coli PRE, the amount of dimer monomerization, assayed by the method of Sutherland and Chamberlin (11), is decreased. (See Fig. 1).



The inhibition of PRE activity shown in Fig. 1 could result from either interference with the binding of the enzyme to a dimer site (the "dark" reaction) or interference with the photochemical reaction. Thus we measured the effect of Nt on the ability of PRE to bind uvDNA using a filter binding assay (12). The presence of Nt decreased the ability of PRE to bind uvDNA as shown in Fig. 2. Since Nt binds in the minor groove of the DNA, these results support a model in which the PRE interacts with the carbonyl groups at the 2



positions of the pyrimidine rings. This conclusion must be regarded as preliminary, however, until we have data on the effects of other probes known to occupy the major as well as the minor grooves of DNA.

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PHOTOSENSITIZED SPLITTING OF THYMINE DIMERS IN DNA BY PEPTIDES AND PROTEIN CONTAINING TRYPTOPHANYL RESIDUES

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ABSTRACT Thymine dimers formed in DNA by UV irradiation can be split by irradiation at 300 nm in the presence of photosensitizers containing an indole ring : serotonin and 5-methoxytryptamine, an oligopeptide containing a tryptophyl residue (Lys-Trp-Lys), and a protein which exhibits a specificity for single stranded nucleic acids (protein coded for by gene 32 of phage T4).

The mechanism of this photosensitized reaction has been investigated using different methods : fluorescence spectroscopy at room and at low temperature, flash photolysis, effect of electron scavengers, ionic strength dependence. In order to act as a photosensitizer, the indole-containing compound must be bound to UV irradiated DNA and stacked with nucleic acid bases and thymine dimers. Dissociation of the complex inhibits the reaction. The splitting arises as a consequence of an electron transfer from the excited indole ring to the dimer. In all cases, the splitting of thymine dimers regenerates intact thymine bases.

INTRODUCTION

Pyrimidine dimers formed in DNA after UV-irradiation can be split either by further irradiation at short wavelengths (1) or by enzymatic photoreactivation (2). This enzymatic process requires light in the wavelength range 300-500 nm where neither DNA nor dimers absorb. Whether the absorption bands corresponding to the action spectrum of the photoreactivating enzyme (PRE) are due to an intrinsic chromophore or produced only by interaction of PRE with its substrate (DNA containing pyrimidine dimers) is still a mat-

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ter of controversy. It appears that these two different situations are encountered in different systems.

Several years ago we showed that freezing an equimolar aqueous solution of thymine dimers (cis-syn) and tryptophan led to a quenching of the tryptophanyl fluorescence (3). Previous work had shown that under these experimental conditions mixed aggregates were formed (4). Irradiation of the frozen mixtures was accompanied by a splitting of thymine dimers and a recovery of thymine monomers as shown by absorption spectroscopy and chromatographic analysis of the reaction products. To prevent the reformation of thymine dimers by direct absorption, we used tryptophan derivatives such as 5-hydroxytryptophan whose absorption spectrum was sufficiently red-shifted so that irradiation could be carried out outside the thymine absorption band (above 300 nm). Under these conditions thymine dimer was quantitatively converted into thymine and the tryptophan derivative was recovered in an intact form after the reaction. This result clearly indicated that the reaction was photosensitized by the tryptophan derivative and that the photosensitizer was not consumed during the reaction (3).

A mechanism was proposed to explain both the quenching of tryptophan fluorescence and the photosensitized splitting of thymine dimers. These two phenomena were ascribed to an electron transfer process occuring from the singlet excited state of the indole ring to thymine dimers, followed by splitting of the dimer and tunnelling back of the electron to the indole ring according to the following scheme (5)

$$Trp^{*} + T\langle\rangle T \rightarrow [Trp \ldots T\langle\rangle T]^{*}$$

$$[Trp^{+\cdot} \downarrow \quad T\langle\rangle T^{-\cdot}]$$

$$Trp + T + T \leftarrow [Trp^{+\cdot} \ldots T^{-\cdot} \ldots T]$$

This scheme applies to frozen aqueous solutions where Trp and T<>T are stacked in mixed aggregates. In fluid solution the situation can be more complicated since electrons photoejected from the excited indole ring might react with T<>T. As well there is a possibility of excimer formation ([Trp ... T<>T]*) or ground-state complex formation (3).

In order to determine whether a tryptophan-photosensitized splitting of pyrimidine dimers could occur under experimental conditions closer to biological conditions we

investigated the photochemical behavior of the complexes formed by UV irradiated DNA and an oligopeptide or a protein containing tryptophan residues.

RESULTS AND DISCUSSION

The experimental conditions used in this work have been already described (6-8). A tryptophan-containing peptide Lys-Trp-Lys was shown to bind to nucleic acids. Two complexes were formed both involving electrostatic interactions. But only one of them involved a stacking interaction of the tryptophanyl ring with nucleic acid bases. In the other one the tryptophanyl residue was not interacting with bases. We showed that the stacking interaction was strongly favored in single stranded as compared to double stranded DNA. As expected pyrimidine dimer formation in native DNA enhanced the stacking interaction (7, 8). When the complex formed by Lys-Trp-Lys with UV-irradiated DNA was further irradiated a splitting of thymidine dimers was observed leading to restoration of thymine bases as shown by chromatographic analysis of hydrolysates of ^{14}C thymine-labeled DNA. However our first experiments were conducted with broad band irradiation ($250 < \lambda < 400$ nm). Therefore it could be argued that in fact the tryptophanyl residue was preventing the reformation of thymine dimers split by direct UV irradiation. As a matter of fact it was expected that tryptophan close to a thymine base would prevent dimer formation since both the singlet and triplet states of thymine are very efficiently deactivated by a stacked tryptophan molecule (4, 9). Moreover a tryptophan inserted between two thymines would obviously prevent dimer formation. In order to show that the splitting of thymine dimers was really a photosensitized reaction we conducted an experiment where irradiation was carried out at wavelengths longer than 295 nm. DNA was preirradiated with UV light ($250 < \lambda < 300$ nm) until 15 % of its thymines were converted into dimers. This irradiated DNA (3×10^{-5} M) was mixed with Lys-Trp-Lys (10^{-4} M) and the mixture was irradiated for 6 hours at wavelengths above 295 nm ($295 < \lambda < 390$ nm). The content of thymine dimers decreased from 15 % to 5.8 % (61 % splitting). Native DNA (3×10^{-5} M) was irradiated alone under the same conditions (6 hours, $295 < \lambda < 390$ nm) : 3.1 % of the thymine was converted into dimers. It is very unlikely that under these conditions thymine dimers

can be split by direct absorption. The splitting of dimers in the presence of Lys-Trp-Lys must therefore be attributed to a photosensitized reaction.

These observations were extended to a protein known to have a strong specific and cooperative binding to single stranded DNA, namely, the protein coded for by gene 32 of phage T4. This protein contains 5 tryptophanyl residues and it was shown that the fluorescence of this protein was quenched upon binding to single stranded DNA (10, 11). When a mixture of gene 32 protein and UV irradiated DNA was further irradiated a splitting of thymine dimers was observed (5, 11). This splitting was still observed when the irradiation was carried out at wavelengths longer than 295 nm. Since the gene 32 protein is very photosensitive (5) the thymine dimer content of the DNA increased again when the mixture of gene 32 protein and UV irradiated DNA was irradiated for long periods of time. This phenomenon was shown to be correlated with the photochemical modification of the tryptophanyl residues which ultimately led to a complete loss of the protein fluorescence (5). If more protein was added more dimers were split. For example a sample of UV irradiated DNA (7.2×10^{-6} M) containing 20.5 % of total thymine converted into dimers was irradiated in the presence of 7.7×10^{-7} M gene 32 protein. The percentage of thymine dimers decreased to 11.8 % after 10 minutes irradiation at wavelengths longer than 280 nm (42 % splitting). After 10 more minutes of irradiation, this value increased to 13.6 %. An equivalent amount of gene 32 protein was then added to the sample and the mixture irradiated again for 10 minutes. The percentage of thymine dimers decreased from 13.6 to 9.6 % (29 % splitting).

The percentage of thymine dimers split by gene 32 protein also increased with the initial gene 32 protein concentration as shown in table 1

Table 1 : effect of gene 32 protein concentration on the splitting of thymine dimers in DNA containing 23 % of thymines converted into dimers. DNA concentration : 6.5×10^{-6} M. Irradiation conditions : 10 minutes, $280 < \lambda < 390$ nm.

gene 32 protein concentration (M)	% radioactivity in the peak of thymine dimers
0	23
2.5×10^{-7}	15
4.9×10^{-7}	11.5
1.2×10^{-6}	9.0

CONCLUSION

The results summarized above show that the splitting of thymine dimers in DNA can be photosensitized by oligopeptides and proteins containing tryptophan. The mechanism of this reaction most probably involves an electron transfer from the excited indole ring to the dimer. A similar conclusion was reached by Chen et al. (12) who showed that indole derivatives and proteins containing tryptophanyl residues were able to photosensitize the splitting of uracil dimers. These authors showed that a protein containing no tryptophan (ribonuclease A) was unable to photosensitize the splitting of uracil dimers. The electron transfer mechanism requires a very close proximity of the thymine dimer and the indole ring. It is expected that this reaction will take place only when the tryptophanyl residues of the protein are involved in stacking interactions with the nucleic acid bases. This might be the case for those proteins which selectively bind to single stranded nucleic acids (and which contain tryptophanyl residues). However the photosensitized splitting was not observed at wavelengths longer than 320 nm. This means that this reaction cannot account for the action spectrum of the photoreactivating enzymes above 320 nm (This does not exclude the possibility that the mechanism of the splitting reaction might be the same, namely, electron transfer to (or from) thymine dimers).

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EVIDENCE FOR PHOTOENZYMATICALLY REPAIRABLE, LETHAL "NONDIMER" PHOTOPRODUCTS FORMED IN *E. COLI* CELLS BY NEAR UV LIGHT[1]

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ABSTRACT The completely dark-repair deficient, non-photorepairable *E. coli* strain, CSR 603, is inactivated by 254-nm radiation at a 300-700 times higher rate than the non-photorepairable dark-repair proficient strain H/r 30. For 365-nm radiation the corresponding inactivation rates differ by a factor of only 5-10, indicating that at this wavelength 10-20% of lethal photoproducts in H/r 30 are not dark-repairable. Thus they must be different from cyclobutane dipyrimidines formed after 254-nm irradiation, of which only less than 1% are not dark-repairable. From the comparison of inactivation of H/r 30 (*phr*$^-$) with H/r 30-R (*phr*$^+$) cells after 365-nm exposure, it is nevertheless evident that the majority of these non-dark repairable photoproducts can be photoenzymatically repaired.

INTRODUCTION

Photoreactivation (PR) after UV damage and the underlying process of photoenzymatic repair is usually the result of monomerization *in situ* of cyclobutane dipyrimidines ("dimers") present in UV-irradiated DNA (1,2). However, the following study of survival kinetics indicates the existence of still other photoenzymatically repairable UV photoproducts in cells lethally damaged by 365-nm radiation.

METHODS

The survival of three pairs of *E. coli* strains with different dark repair potentials, *viz.*: H/r 30 (*phr*$^-$) and H/r 30-R (*phr*$^+$) (Ref. 3); H_s 30 (*phr*$^-$) and H_s 30-R (*phr*$^+$)

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(Ref. 4); CSR 603 (*phr*$^-$) (Ref. 5) and AB 2480 (*phr*$^+$) (Ref. 6); was investigated after irradiation at 254 nm (from a GE "germicidal" lamp) or at 365 nm (from a B&L grating monochromator). Except for the photorepair (*phr*) locus, the two strains of each pair have identical or similar genotype. PR after 254-nm irradiation was achieved by illumination with "white" fluorescent light at 37°C, while inactivation at 365 nm already includes the PR treatment, as this wavelength is about maximally effective in photorepair of *E. coli*.

RESEULTS AND DISCUSSION

Inactivation by 254-nm Radiation. As expected, cell survival after 254-nm exposure without PR treatment did not differ appreciably within each pair of strains. The mean inactivation fluences ($F_{0.37}$) for the exponential portions of survival curves are 0.02 J·m^{-2} for the *uvrA recA* strains CSR 603 and AB 2480; 1.0 J·m^{-2} for the two *uvr*$^-$ strains H_S 30 and H_S 30-R; and 6.25 to 15 J·m^{-2} for the dark-repair-proficient strains H/r 30 and H/r 30-R, depending on which region of the shouldered curve is considered. Thus the latter are 300-750 times more UV-resistant than the two completely dark-repair deficient strains. PR treatment after UV exposure resulted in no PR of the *phr*$^-$ strains, but in photoreactivable sectors (*i.e.* probabilities for lethal lesions of being photorepaired) of 0.6 to 0.8 in all *phr*$^+$ strains.

Inactivation by 365-nm Radiation. Like 254 nm, 365-nm radiation produces pyrimidine dimers in *E. coli* DNA (7) and inactivates and (simultaneously) photoreactivates dark-repair deficient cells (8). Figure 1 shows that within each pair the *phr*$^+$ strain survives 365-nm exposure much better than *phr*$^-$ strain, indicating that lethal damage by 365 nm is photoenzymatically repaired by the same wavelength. The photoreactivable sectors, ranging from about 0.5 to 0.7, resemble those observed for 254-nm damage.

It is evident from the figure that after 365-nm exposure the sensitivity differences between the 3 pairs of strains are much smaller than after 254-nm exposure. For example, CSR 603 (*phr*$^-$) is only 5-10 times more sensitive to 365 nm than the repair-proficient strain H/r 30 (*phr*$^-$), while there is a 300 to 750-fold difference at 254 nm. Since these strains are not photoreactivable, the observed differences indicate that in H/r 30 cells at least 99.7% of lethal lesions are dark-repaired after 254-nm exposure, but only 80-90% after 365-nm exposure. Considering that the majority of primary 254-nm photoproducts in DNA are "dimers", their almost com-

plete dark repair means that the 10-20% unrepaired lethal lesions after 365-nm exposure can *not* be dimers of the types produced by 254 nm irradiation. Since they are nevertheless photorepairable (as evidenced by the much higher survival of H/r 30-R cells, compared with H/r 30 *phr*$^-$), the *E. coli* photorepair enzyme must be capable of abolishing other lethal photoproducts besides dimers.

Similar considerations apply for the pair of partially dark-repair deficient strains (lacking excision repair but being proficient for *recA* controlled repair). The figure shows that H_s 30 *phr*$^-$ cells require only 2-3 times more 365-nm fluence than CSR 603 cells for the same extent of inactivation, in contrast to a 50-fold difference in the $F_{0.37}$'s at 254 nm. The considerable photorepair of 365-nm damage, displayed by the different survival of H_s 30 and H_s 30-R cells, suggests that the fraction of photorepairable "non-dimer" damage after 365-nm exposure may even be higher than appears from the H/r 30 results.

Controls. An argument against this interpretation would be that reduced dark repair of 365-nm lesions in H/r 30 cells

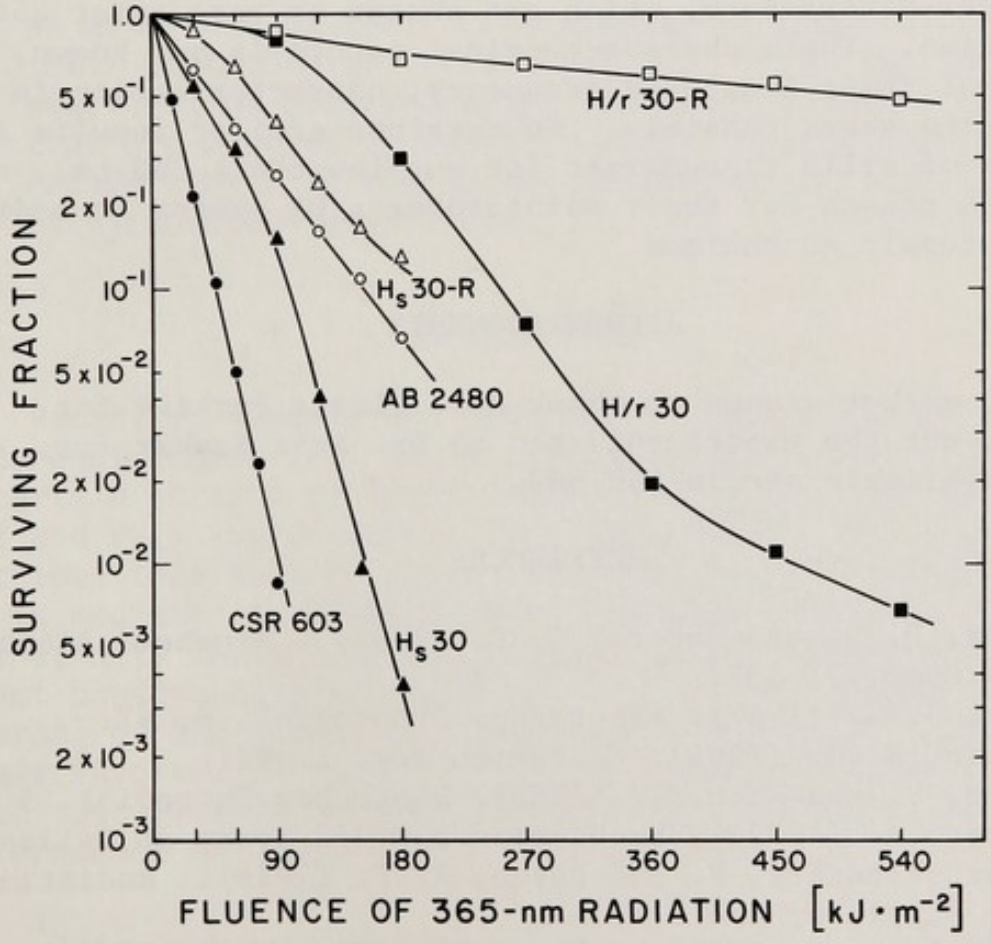

Figure 1. Survival of *phr*$^-$ cells (filled symbols) and *phr*$^+$ cells (open symbols) after exposure to 365-nm radiation (geometric means of 3 experiments).

is a consequence of damage to components of the dark repair systems themselves. However, this possibility was ruled out by the following experiments: H/r 30 and H/r 30-R cells were exposed to 200 $KJ \cdot m^{-2}$ of 365-nm radiation, leading to average survivals of 23% and 88%, respectively. The "predamaged" cells were then exposed to 254-nm radiation and their survival compared with that of non-predamaged cells of the same strain. In all three experiments of this kind the 254-nm inactivation was essentially the same for predamaged and non-predamaged cells. This would not be significant in the case of H/r 30, since the surviving cells after 365-nm irradiation can anyway be expected to be those whose dark repair systems remained undamaged. However, in the case of H/r 30-R, a major fraction of the cells, namely (88%-23%)/88%, survive as a result of PR. If in these cells one of the dark-repair systems were rendered non-functional, subsequent 254-nm irradiation would cause a very steep initial decline of the survival curve, which was not observed.

In conclusion, our data indicate the presence of photoenzymatically repairable, non-dark-repairable photoproducts after near-UV exposure, which are absent or rare after 254-nm irradiation. Their physico-chemical nature is not known, but in view of their indicated frequency, characterization in these terms seems feasible. We obtained similar results after exposure of cells to sunlight (at wavelengths > 360 nm), suggesting a reason for their maintaining a PR system in addition to dark repair mechanisms.

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PHOTOREACTIVATION OF *Escherichia coli* IRRADIATED WITH IONIZING RADIATION[1]

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ABSTRACT By comparing the results for a *uvrA recA* and a *uvrA uvrB recA phr* strain, we conclude that the photoreactivation after ionizing irradiation involves the same enzyme that is responsible for the photoreactivation of UV-induced cyclobutadipyrimidines. A comparison of the photoreactivable sectors in a *uvrA recA* strain after UV and ^{137}Cs-γ-irradiation indicates that 10 krads of γ-radiation produces about 0.07 Jm^{-2}-equivalents of 254 nm-induced photoreactivable damage. After 400 krads, an acid hydrolysate of the isolated DNA revealed the presence of thymine-containing cyclobutadipyrimidines, as evidenced by their chromatographic properties and their photochemical reversibility. Thus, the photoreactivation observed after ionizing radiation in strains that are blocked in excision repair and in postreplication repair is due to the production of trace amounts of cyclobutadipyrimidines.

INTRODUCTION

Recently, Myasnik and Morozov (1) demonstrated that photoreactivation (PR) after γ-irradiation can be observed in certain strains of *Escherichia coli*, e.g., *uvrA recA* of K-12 and B_{s-1} (*uvrB lexA*). In the same work, the authors described that this PR is not produced by preillumination and is mediated by a temperature-dependent process, suggesting that it acts through photoenzymatic repair rather than by indirect photoreactivation (2) or the direct photochemical reversal of the damage (3). The only known photoenzymatic repair involves the use of photoreactivating enzyme (PRE), which specifically monomerizes cyclobutadipyrimidines in the presence of light

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(310-480 nm). (For reviews see 4-6). Therefore, two major questions arose: 1) does the observed PR after ionizing irradiation involve the same PRE as after UV irradiation, and 2) what is the γ-ray-induced lethal damage that is removed after exposure to light? The present work presents evidence that cyclobutadipyrimidines are produced by ionizing radiation, and that the observed PR after ionizing irradiation involves the same PRE as after UV irradiation.

Photoreactivating Enzyme is Required for Photoreactivation After Ionizing Irradiation. We have compared K-12 strains AB2480 (*uvrA recA*) and SR362 (*uvrA uvrB recA phr*) for their ability to be photoreactivated after γ-irradiation.

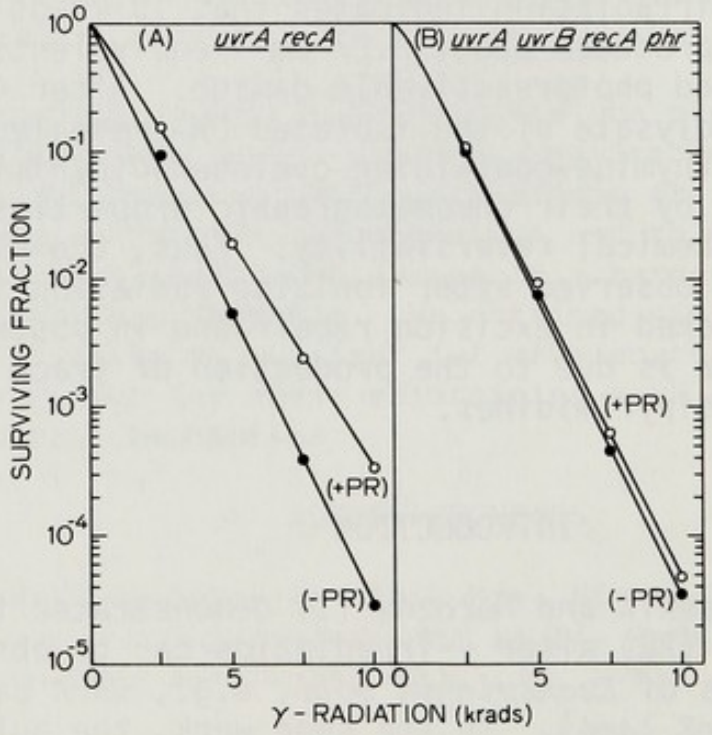

FIGURE 1. The effect of post-illumination with near-UV radiation on the survival of AB2480 (*uvrA recA*) and SR362 (*uvrA uvrB recA phr*) after γ-irradiation. Stationary phase cells grown in YEP (1) were suspended in DTM buffer (9) at 1 x 10^8 cells/ml, and irradiated with ^{137}Cs-γ-rays at room temperature without aeration. Samples were either kept in the dark (-PR), or post-illuminated (+PR) with 15-W Daylight fluorescent lamps for 3 min at a distance of 3 cm above the light source with a glass plate in between.

As shown in Fig. 1, Phr^+ AB2480 showed PR after γ-irradiation, as reported by others (1,7). In contrast, Phr^- SR362, which showed no detectable PR after UV irradiation, failed to show any detectable PR after γ-irradiation. This indicates that the same PRE is responsible for the observed PR after ionizing irradiation as well as after UV irradiation. A comparison of the photoreactivable sectors in strain AB2480 after UV irradiation (data not shown) and after γ-irradiation (Fig. 1) reveals that 10 krads of ^{137}Cs-γ-radiation produced about 0.07 J m^{-2}-equivalents of 254 nm-radiation-induced photoreactivable damage.

Production of Dimers by γ-Irradiation. Since the only substrate known for the PRE is cyclobutadipyrimidines (see 6), and there is material chromatographing similar to pyrimidine dimers after ionizing irradiation (8), we examined the possibility of dimer production by ionizing radiation. Strain SR73, a *thy* derivative of AB2480, was labeled in DTM-glucose medium (9) in the presence of 100 μCi/ml of ^3H-thymidine, and was γ-irradiated at 4°C in DTM buffer with 400 krads. The DNA was extracted, hydrolyzed with trifluroacetic acid at 175°C for 75 min, and chromatographed on Whatman #1 paper using: (A) *n*-butanol: acetic acid: H_2O (40:6:15), (B) *n*-butanol: H_2O (43:7), (C) isopropanol: NH_4OH: H_2O (7:1:2), and (D) *sec*-butanol saturated with H_2O. Authentic T<>T and U<>T (via deamination during acid-hydrolysis of C<>T) were isolated from UV-irradiated ^{14}C-labeled SR73 and used in parallel runs to locate the regions corresponding to T<>T and U<>T. In assays for T<>T, radioactivity eluted from the T<>T region in solvent (A) was rechromatographed in the same solvent to eliminate the streaking of thymine in the T<>T region. Of the isolated material, about 25% could be reversed to thymine by irradiation at 254 nm. In the subsequent development with solvent (B), material having Rf 0.2 can be separated from the T<>T region (Rf 0.11). Using solvent (C), material isolated from the T<>T region (Rf ∿0.57) can be further separated from other contaminating materials (Rfs ∿0.31 and 0.42, respectively). The isolated material that chromatographs identically with T<>T in solvents (A), (B), and (C), also chromatographs identically with T<>T in solvent (D), and is completely reversed to thymine upon exposure to UV radiation (data not shown). This material, therefore, can be confidently identified as T<>T.

In assays for U<>T, material corresponding to the U<>T region from solvent (A) was similarly chromatographed with solvents (B) and (C), as was done for T<>T. There is radioactive material that chromatographs as U<>T in solvents (B) and (C), but the separation of U<>T from other contaminating

materials is not satisfactory, and its yield can't be quantitated with confidence.

The purified T<>T after 400 krads represents 7.4×10^{-5} of the total ^{3}H-thymine radioactivity. Therefore, 400 krads of ^{137}Cs-γ-radiation is equivalent to 2.6-3.7 J m^{-2} of 254 nm-radiation in producing cyclobutadipyrimidines, if we take the initial rate of production of T<>T in the DNA of UV-irradiated cells as $2.0\text{-}2.75 \times 10^{-5}$ radioactivity in T<>T/total radioactivity as T per J m^{-2} (10,11). This result is in good agreement with our *in vivo* estimate based upon survival curves. (10 krads=0.07 J m^{-2} of 254 nm radiation).

It is understandable why PR after ionizing radiation can only be maximally detected in *E. coli* K-12 *uvrA recA* and B_{s-1}; these strains show no shoulder in their UV-inactivation curves. Likewise, it is to be anticipated that no detectable PR can be demonstrated in *uvrB* or wild-type cells, because the γ-radiation-dose used for detecting PR has an equivalent UV-effect that corresponds to the shoulder region of the UV-inactivation curves in these strains.

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ULTRAVIOLET-LIGHT TRIGGERED DISAPPEARANCE OF PHOTOREACTIVATING ENZYME[1]

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ABSTRACT UVS 1 is a mutant of Chlamydomonas reinhardi defective in the dark-repair (presumably excision repair) of pyrimidine dimers from nuclear DNA. All of the pyrimidine dimers in nuclear DNA can be repaired upon exposure to photoreactivating light immediately after irradiation. However, none of the dimers are photoreactivable if the cells are incubated for 24 hr in the dark in growth medium. Pyrimidine dimers induced in chloroplast DNA can be photoreactivated even after 24 hr incubation in the dark. Assay for photoreactivating enzyme in extracts shows that about 80% of the photoreactivating enzyme activity disappears after incubating UV-irradiated cells in the dark for 24 hr. No significant disappearance of enzyme activity occurs in the case of unirradiated cells. It is hypothesized that the residual photoreactivating enzyme found in incubated, irradiated cells represents chloroplast enzyme and that UV-irradiation triggers the disappearance of nuclear photoreactivating enzyme.

INTRODUCTION

Chlamydomonas reinhardi has several attributes making it an attractive model eukaryotic cell for the study of DNA repair: ease in growth and handling, availability of repair-deficient mutants (1,2) and the possibility of examining repair of organelle DNA. Using a UV-specific endonuclease to monitor levels of pyrimidine dimers, we have shown that wild-type cells remove nearly all the dimers induced in nuclear DNA with 50 J/m^2 of 254 nm light upon incubation for 24 hr in the dark in growth medium (3). The UV-sensitive mutant, UVS 1, is completely deficient in this dark-repair pathway. Dimers induced in chloroplast DNA can be repaired either by photoreactivation or by a dark-repair pathway, although the

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latter mode is less efficient (4). This chloroplast DNA dark-repair pathway is also present in UVS 1, suggesting that nuclear and chloroplast dark-repair pathways are under separate genetic control.

The present communication shows that ultraviolet irradiation of UVS 1 cells triggers the disappearance of nuclear photoreactivating enzyme upon incubation in growth medium in the dark.

METHODS

The procedures for labeling nuclear (3) and chloroplast (4) DNA and analysis of dimers using UV-specific endonuclease are detailed in our earlier publications. The photoreactivating enzyme assay consists of incubating [^{3}H] DNA from T7 phage irradiated with a fluence of 1500 J/m^2 with Chlamydomonas extract in a solution of 30 mM glycyl-glycine, pH 7.0, 90 mM NaCl, and 1 mM dithiothreitol for 30 min at 30 C. A unit of enzyme activity is defined as the amount of protein that catalyzes the monomerization of 1 pmole of pyrimidine dimer per hr.

RESULTS AND DISCUSSION

Pyrimidine dimers induced in nuclear DNA by UV can be completely repaired if the cells are exposed to photoreactivating light immediately after irradiation (Figure 1, upper and lower left panels). However, if the irradiated cells are incubated for 24 hr in the dark in growth medium, none of the dimers can be removed by photoreactivation (Figure 1, upper and lower right panels). Two possible explanations for this observation were considered: one, the dimers were altered during the dark incubation so that they were not recognized by the photoreactivating enzyme but were still susceptible to the UV-specific endonuclease; two, photoreactivating enzyme was no longer active.

To test the first possibility, UVS 1 was irradiated (50 J/m^2), incubated in the dark for 24 hr, given a second fluence of 254 nm light (75 J/m^2) producing additional dimers and subjected to photoreactivating light. None of the dimers were repaired by photoreactivation. Thus, it appears unlikely that lack of photoreactivation is due to alteration of the dimers during the dark incubation.

To test the second possibility, extracts were made of irradiated cells that had been incubated in the dark for 24 hr and the amount of photoreactivating enzyme was measured and compared to controls. The results are summarized in

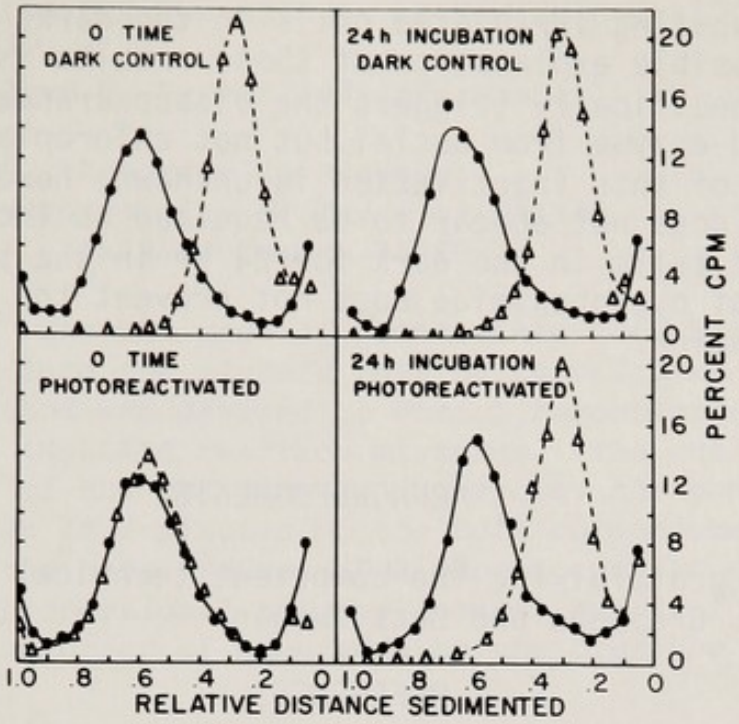

FIGURE 1. Sedimentation analysis of DNA in alkaline sucrose gradients. Chlamydomonas was exposed to photoreactivating light either immediately after irradiation (50 J/m^2) or 24 hr after incubating the irradiated cells in the dark in growth medium. DNA was extracted and the presence of pyrimidine dimers determined by the susceptibility of the DNA to hydrolysis by UV-specific endonuclease. Sedimentation was from right to left. ●——● control; Δ- - - -Δ incubated with UV-specific endonuclease.

Table I. Incubation of irradiated cells in growth medium for 24 hr in the dark leads to the disappearance of approximately 80% of the photoreactivating enzyme activity. The residual photoreactivating enzyme activity may well be derived from chloroplast as chloroplast DNA remains photoreactivable

TABLE I

PHOTOREACTIVATING ENZYME ASSAYS[a]

Incubation time	Unirradiated	Irradiated
0	140	163
24 hr	120	23

[a]Activity is expressed as units/mg protein

after incubating irradiated cells in the dark.

A possible explanation of these results is that UV-irradiation specifically triggers the disappearance of photoreactivating enzyme from nuclei but not chloroplasts. The mechanism of this inactivation is unknown; however, protein synthesis does not appear to be required as incubation of irradiated cells in the dark for 24 hr in the presence of 10 ug/ml of cycloheximide does not prevent the loss of photoreactivation.

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I am grateful for the competent technical assistance of Carolyn S. Greimann and Betty Hogan.

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CLONING THE PHR GENE OF ESCHERICHIA COLI[1,2]

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ABSTRACT The phr gene of Escherichia coli has been inserted into the plasmid pMB9 by in vitro recombination A new method was devised to enrich recombinant DNA molecules in in vitro reaction mixtures. The published map location of the phr gene is incorrect, the gene actually lying near 15.7 minutes on the coli chromosome. Production of the gene product (DNA photolyase, EC 4.1.99.3) is roughly proportional to gene dosage, and is further increased an order of magnitude by the presence of the purA mutation.

INTRODUCTION

The recently developed methods for inserting genes into independently replicating vehicles (plasmids or bacteriophage chromosomes) by in vitro DNA recombination offer powerful means for purifying a gene and amplifying its product, which open the way to important studies of gene structure and gene function. These methods involve (a) cleaving the gene-bearing and vehicle DNA's with a restriction endonuclease which makes staggered, double-stranded cuts through a specific palindromic base pair sequence, (b) annealing and ligating mixtures of these DNA's, (c) inserting the resulting recombinant plasmids into cells and (d) selecting for those carrying the desired gene (1). The genes controlling major DNA repair processes have not previously been cloned in this manner. We have recently inserted the phr gene of Escherichia coli into the modified ColE1 plasmid pMB9 (containing only a single site for cleavage by EcoR1 endonuclease, plus a gene for tetracycline resistance, which is useful for its selection)

[1]This work was supported by Research Grant R 01 GM16547 from the U.S. Public Health Service.

[2]All work with recombinant plasmids was conducted under conditions of physical containment P1, and biological containment EK1, in accordance with biohazard regulations.

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(2), and have carried out preliminary investigations of the recombinant plasmid. The process required the solution of two problems, having interest of their own.

RESULTS

Map location of the *phr* gene. We initially intended to use a purified F' plasmid, carrying the appropriate region of the *E. coli* chromosome as a source of the *phr* gene. However, unexpected difficulties finally forced us to recognize that the gene is not located as published (3,4), between *gal* and *attλ*. A series of cell strains variously deleted in the *gal* to *attλ* and neighboring regions (*viz.*, RW361 (5), SA291 (6), C218 (6)) all tested *phr*$^+$ for colony formation and survival of irradiated infecting T4 phage. Further, 112 *gal*$^-$ deletion mutants, made from *E. coli* DC515 (λCI857 *xis red*) (a heat-inducible lysogen of an excision-defective λ, in which the rare survivors of induction are deleted around *attλ*) (D. Court, private communication), all tested *phr*$^+$. Conjugation of a *phr*$^-$ strain with two Hfr strains (EC2701 and EC2702), both of which donate gene sequences beginning within the *gal* gene, but running opposite directions on the map (7), showed that the gene lies counterclockwise from *gal*, (i.e., away from *attλ*) a conclusion confirmed by transfer of F' plasmids carrying different chromosomal regions. The frequency of *phr*-*gal* co-transduction by P1kc corresponded to ∿1 min on the map (8), which would place *phr* around 15.7 minutes. Independent work by D.A. Youngs and K.C. Smith has led to identical conclusions (private communication).

Enrichment of Recombinant DNA. The F' in our possession carrying the *phr* gene (F153, in Coli Genetic Stock Center Strain #5209) is too large for efficient separation from cellular DNA fragments after cell lysis. Recombining these unpurified fragments with pMB9 (a "shotgun" experiment), did not lead to selection until a preliminary enrichment for recombinants, based on the photolyase membrane binding assay of Madden *et al*. (9), was applied.

DNA photolyase binds strongly to UV-irradiated DNA, from which it is released, with repair of the UV damage, by exposure to light (10). Proteins bound to DNA will attach it temporarily to nitrocellulose membranes (11). Consequently, the *phr*-bearing *E. coli* DNA was irradiated with 80 J/m^2 254-nm UV, either before or after restriction endonuclease treatment, and added at 10 μg/ml to 4 μg/ml pMB9 DNA. After annealing and ligating, it was mixed with an excess of highly purified yeast photolyase (12) at 4 μg/ml, and passed through a Schleicher and Schuell, B-6 nitrocellulose membrane filter

using only red or yellow iblumination. DNA containing the irradiated E. coli sequences carried bound yeast photolyase, and tended to remain on the filter. The large number of plasmid molecules which had recircularized without incorporating coli DNA simply passed through the filter. Placing the rinsed filter in a sterile vial, and exposing it to 1 hour of black light illumination (five GEF:478-B1B tubes at 30 cm) released and repaired the bound DNA, and adding E. coli made competent for transformation by the procedure of Cohen et al. (13), allowed the freed DNA to enter cells. Reconstruction experiments, using two differently marked plasmids (PSF2124, with resistance to ampicillin, and pBR312, with an additional resistance to tetracycline), showed that this procedure actually enriches about 350-fold for transformation by DNA of the selected (UV-irradiated) plasmid in a mixture. Stringent selection for the cells which have taken up DNA containing pMB9, is provided by the resulting tetracycline resistance.

Cloning of the phr gene. Applying this procedure to a "shotgun" reaction mixture, and subjecting the transformed culture to two rounds of UV inactivation, plus maximal photoreactivation after exposure to tetracycline, gave a population with about half the cells containing a phr-bearing plasmid. One clone was selected for characterization.

The plasmid, pCSR604, isolated by the procedure of Guerry et al. (14), gave about $4x10^3$ PHR^+ TET^+ transformants of CSR603 (PHR^--TET^-) per μg DNA. The contour length, visualized by electron microscopy, is about 11.2 μm (molecular weight 22.5 daltons). Upon digestion with EcoR1, it yields 3 fragments: $3.5x10^6$ (the vehicle plasmid pMB9), $7.0x10^6$ and $12.0x10^6$, as indicated by agarose gel electrophoresis. We do not yet know whether the last two fragments are both necessary.

This plasmid normally constitutes about 3.4% of the total cellular DNA, corresponding to 10 copies per cell. It continues its replication in the presence of chloramphenicol. The number of photolyase molecules in cells containing it, measured by the flash illumination method (15), is an order of magnitude above the normal 10-20 for K12 wild type, consistent with the increase in gene dosage.

Nishioka and Harm (16) had shown earlier that the purA mutation increases photolyase production in otherwise normal cells by about an order of magnitude, through a mechanism not understood. When pCSR604 was inserted into a ($recA^-purA^-$) by transformation, the number of photolyase molecules in the resulting strain increased to about 1300 per cell -- nearly

100-fold higher than in wild type cells. Thus the purA effect multiplies the effect of gene dosage on the phr gene product.

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BASE EXCISION REPAIR OF DNA[1]

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ABSTRACT Base excision repair is a specific DNA repair mode in which damaged or inappropriate bases are excised from DNA as the free base. Enzymes that catalyze this reaction are termed DNA glycosylases. The sites of base loss are presumably attacked by apurinic-apyrimidinic endonucleases which create free ends for subsequent exonucleolytic excision of the sugar-phosphate residue. The known DNA glycosylases and apurinic endonucleases are discussed and biological evidence is presented in support of base excision repair in living organisms.

INTRODUCTION

The general term excision repair refers to the enzymatic removal of damaged bases from DNA and has been best characterized in the study of the molecular mechanism of pyrimidine dimer excision (1-5). It has been well demonstrated in a number of prokaryote systems that enzyme-catalyzed breakage of phosphodiester bonds on the 5' side of pyrimidine dimers in DNA is a primary repair event (6-8). The dimers are then removed from DNA as part of an oligonucleotide sequence by nuclease-catalyzed degradation in the 5' → 3' direction (5). In recent years it has become established that damaged or inappropriate bases in DNA can be excised by a different mechanism, in which the primary event appears to be removal of the free base by enzyme-catalyzed hydrolysis of the bond linking the base to the sugar-phosphate backbone of a polynucleotide strand. We have suggested (9, 10) that this repair mode be referred to as base excision repair to differentiate it from the previously described nucleotide excision repair mode.

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ENZYMES IN BASE EXCISION REPAIR

The existence of base excision repair in living organisms is suggested in part by the isolation, purification and characterization of two specific classes of enzymes; DNA glycosylases and apurinic endonucleases.

a) DNA glycosylases catalyze the hydrolysis of the bond linking either purines or pyrimidines to the deoxyribose moeity in DNA. This bond has been traditionally referred to as a N-glycosidic bond, hence the original designation of this class of enzymes as DNA N-glycosidases (11). However, according to the existing Rules for Biochemical Nomenclature (12), the linkage of the C_1 of a sugar to the N of an aglycone falls under the definition of a glycosylic linkage. Hence, enzymes that catalyze the hydrolysis of this bond in deoxyribonucleic acid are now referred to as DNA glycosylases (W. E. Cohn, personal communication; T. Lindahl, personal communication).

Within this class a number of enzyme activities have been reported. Goldthwait and his colleagues have reported no activity that catalyzes the release of 3-methyladenine and O^6-methylguanine from alkylated DNA (13) and the release of arylpurine derivatives from DNA treated with 7-bromomethyl-12-methylbenz[a]anthracene (14). More recently, Lindahl and his colleagues (15, 16) and Laval (17) have reported the isolation of a 3-methyladenine-DNA glycosylase without any other associated activities from *E. coli* and *M. luteus* respectively.

A clearly distinct DNA glycosylase catalyzes the release of free uracil from DNA or deoxyribonuclear polymers that contain this base. The enzymatic degradation of uracil-containing DNA was initially reported by Wovcha and Warner (18) and by Carrier and Setlow (19). The latter authors used phage PBS2 DNA (which naturally contains uracil instead of thymine) as a substrate. In collaboration with Dr. Ann Ganesan at Stanford University, we originally detected an activity that selectively degraded PBS2 DNA, in a partially purified preparation of phage T4 endonuclease V. We have since carried out further investigations on this activity in extracts of *B. subtilis*, the natural host of phage PBS2, since we were interested in examining the biological relationship between the phage (containing uracil in its DNA) and the host (containing an activity that degrades such DNA).

When an extract of *B. subtilis* is incubated in the presence of EDTA with [^{3}H] uracil-labeled PBS2 DNA, or with [^{3}H] thymine-labeled *E. coli* DNA, a significant fraction of the label is released from the former substrate, but not from the latter (Fig. 1) (20). Chromatographic analysis of the acid-soluble fraction from such a reaction reveals that all of the radioactivity is in the form of free uracil. None is detected as dUMP or as Udr (9, 20). In an independent study with ex-

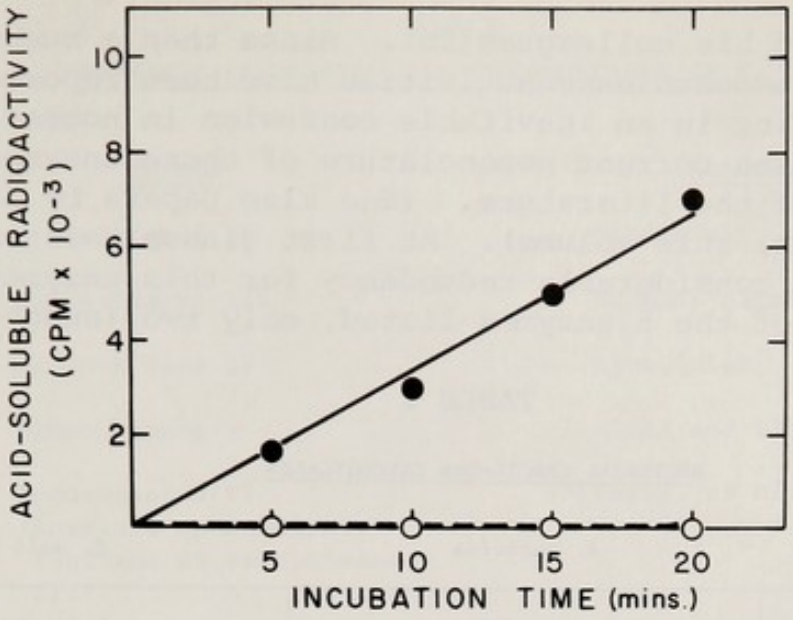

FIGURE 1. Degradation of PBS 2 DNA (closed circles) and T7 DNA (open circles) by extracts of *Bacillus subtilis*. See reference 20 for experimental details.

tracts of *E. coli*, Lindahl demonstrated an activity that in the presence of EDTA releases free uracil (derived by the deamination of cytosine) from *E. coli* DNA (11). Subsequent studies on purified preparations have demonstrated that both the *E. coli* (21) and the *B. subtilis* enzymes are true DNA glycosylases, i.e., free uracil is released from DNA or poly (dU) without concomitant cleavage of phosphodiester bonds. Table I lists some of the comparative properties of the two enzymes.

Infection of *B. subtilis* with phage PBS2 results in a very rapid loss of ura-DNA glycosylase activity (20, 23, 24). This result is dependent on active protein synthesis following infection, suggesting that the phage codes for or induces the synthesis of a specific inhibitor of the enzyme. The inhibitor is detectable in extracts of phage-infected cells (20, 23) and has been purified close to homogeneity in our laboratory (R. Cone and E. C. Friedberg, unpublished observations). It is a relatively small protein that is stable to boiling and its mechanism of inhibition of the host ura-DNA glycosylase is currently under detailed investigation. Interestingly, the inhibitor is also active against both the *E. coli* enzyme and that present in human cells in culture. (J. Duncan, K. H. Cook and E. C. Friedberg, unpublished observations).

b) Apurinic endonucleases Members of this class of enzymes have been shown to attack both apurinic and apyrimidinic sites in DNA, but for want of a more descriptive inclusive name are referred to simply as apurinic endonucleases. Endonuclease activity specific for sites of purine loss in DNA (apurinic sites) was first reported by Hadi and Goldthwait (25)

and by Verly and his colleagues(26). Since then a number of other apurinic endonuclease activities have been reported in *E. coli*, resulting in an inevitable confusion in nomenclature. Table II lists the current nomenclature of these enzymes based on our review of the literature. (See also papers by Linn, Verly, and Weiss, this volume). At first glance this catalogue would suggest a considerable redundancy for this enzymatic function. However of the 5 enzymes listed, only two (endonuclease

TABLE I

BACTERIAL URACIL-DNA GLYCOSYLASES

Property	*B. subtilis*	*E. coli*
Molecular weight	∿ 24,000	∿ 24,500
Complexity	No subunits	No subunits
pH optimum	7.3 - 7.8	8.0
Divalent cation	No requirement Not stimulated by Mg^{++} or Mn^{++}	No requirement Not stimulated by Mg^{++} or Mn^{++}
Heavy metals	Co^{++}, Fe^{++} and Zn^{++} inhibit	?
Monovalent cations (50 mM NaCl)	Slight stimulation	Slight inhibition
SH-inhibitors	Insensitive to PCMPSA	Slight inhibition by PCMPSA Insensitive to NEM
Associated enzyme activities	None detected	None detected
Km	1×10^{-9}M for dUMP in poly (dU)	4×10^{-8}M for dUMP in PBS1 DNA
Substrate specificity		
a) Polynucleotides containing uracil	Attacks both double and single stranded substrates	Attacks both double and single stranded substrates
b) Minimal sized oligomer	poly $(dU)_4$	PBS1 DNA < trinucleotides not a substrate
c) Other bases in DNA	No activity	No activity
d) dUTP, dUMP, UdR	No activity	No activity
e) RNA	No activity	No activity

TABLE II

APURINIC (APYRIMIDINIC) ENDONUCLEASES OF *E. coli*

Enzyme	Authors
Endonuclease II	Goldthwait, *et al.*
Endonuclease III	Radman; Gates and Linn
Endonuclease IV	Ljungquist
Endonuclease V	Gates and Linn
Endonuclease VI (Apurinic endonuclease function of exonuclease III)	Verly, *et al.*; Weiss

IV and VI) appear to be exclusively apurinic endonucleases. As indicated previously, endonuclease II is reported to be associated with a DNA-glycosylase function of broad substrate specificity (13, 14). Endonuclease III (27) and endonuclease V (28) are enzymes reported to recognize a wide range of structural damage in DNA including sites of base loss. Hopefully the isolation of mutants defective in each of these enzymes will help resolve whether or not all of the activities listed in Table II represent distinct endonucleases and what their primary biological functions are. At present the only known mutants in a single apurinic endonuclease activity are those defective in the x^{th} gene, which codes for endonuclease VI (exonuclease III) (29). These mutants are abnormally sensitive to methylmethanesulfonate (29) as well as to nitrous acid (30). Since both of these forms of DNA damage produce base changes which are substrates for known DNA glycosylases, these results strongly suggest a role for endonuclease VI in base excision repair in *E. coli*.

None of the endonucleases referred to above have been shown to attack sites of base loss in single stranded DNA. Indeed, it has been specifically reported that neither endonuclease VI (31) nor endonuclease IV (32) of *E. coli* degrade such a substrate. Recently, we have detected an activity that, in the presence of EDTA, catalyzes the degradation of depyrimidinated PBS2 or duprinated *E. coli* single stranded DNA to acid-soluble products (Fig. 2) (T. Bonura and E. C. Friedberg, unpublished observations). Single stranded DNA or poly (dU) with intact bases is not significantly degraded under these conditions of incubation (Fig. 2). The activity is present in a deletion mutant of *E. coli* (BW9101) (29) defective in endonuclease VI. It has no requirement for divalent cation, thereby distinguishing it from endonuclease V. In addition it is inhibited in the presence of tRNA and loses 50% activity

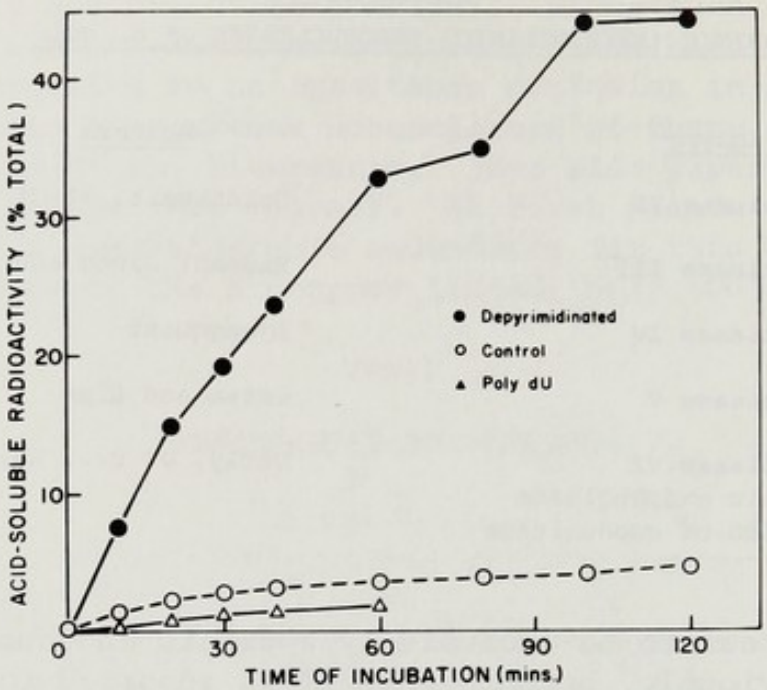

FIGURE 2. Degradation of single stranded depyrimidinated DNA by extracts of *E. coli* BD119 (*ung*$^-$). Reaction mixtures contained crude extract (8-30 µg protein) 5.0 mM $CaCl_2$, 6 mM Tris-HCl buffer pH 7.5 and either [^{32}P] labeled alkali denatured and then depyrimidinated PBS2 DNA (80% uracil removed, closed circles) alkali denatured non-depyrimidinated PBS2 DNA (open circles) or [^{3}H] poly (dU) (triangles).

in the presence of 0.2 M NaCl, or when crude extracts are heated to 50°C for 10 minutes. These features distinguish it from endonuclease IV. Finally, incubation of 9 units of purified endonuclease III (27) (kindly provided by Dr. Stuart Linn) with depurinated M-13 DNA resulted in very limited cleavage of this substrate in our hands. On the other hand incubation of this amount of endonuclease III with a molar equivalent of depurinated form I PM2 DNA resulted in complete conversion of this DNA to the relaxed form. It is conceivable that this enzyme has a specific role in the repair of sites of base loss at or near replication forks in DNA, in which topological considerations preclude binding of other apurinic endonucleases specific for double stranded DNA. The enzyme is currently being purified for further study.

BIOLOGICAL STUDIES IN BASE EXCISION REPAIR

Aside from its occurence in the DNA of phages PBS1 and PBS2, uracil can arise in thymine-containing DNA by incorporation during DNA synthesis or by deamination of cytosine. Concerning the former mechanism, both Konrad and Lehman (33) and

Hochhauser and Weiss (34) independently isolated mutants of *E. coli* defective in dUTPase (*dut*$^-$). The resulting alteration of the dUTP pool is associated with significant incorporation of uracil into DNA during replication (35). Evidence for its removal by a base excision repair mode has been provided by Tye and Lehman (36) who showed that so-called short Okazaki fragments in *E. coli* are probably not replicative intermediates, but repair intermediates that arise from DNA glycosylase-catalyzed removal of uracil, followed by DNA strand breakage (presumably enzyme catalyzed). Short Okazaki fragments are not generated in *dut*$^-$ mutants that are also defective in ura-DNA glycosylase (*ung*$^-$) (36).

As regards the repair of uracil that originates from cytosine deamination, we have observed that *ung*$^-$ mutants isolated by Duncan and Warner (manuscript in preparation) are abnormally sensitive to both nitrous acid (30) and to treatment with sodium bisulfite (R. Simmons and E. C. Friedberg, manuscript in preparation (Fig. 3) Nitrous acid-treated DNA is a rather complex model substrate in which to study the excision repair of uracil since the chemical is known to have pleiotropic effects on DNA (37). On the other hand, evidence in the literature (38) suggests that at least *in vitro*, sodium bisulfite specifically deaminates cytosine under defined experimental conditions. This conclusion is supported by the results of experiments in which we examined the survival of the T series of coliphages following treatment with sodium bisulfite. In our hands the the T-even phages T2, and T4 (which contain hydroxymethylcytosine instead of cytosine in their DNA) (39) are insensitive to inactivation when treated with 0.9 M sodium bisulfite for up to 2 hours. On the other hand the T-odd phages (T3 and T5) as well as a mutant of T4 (T42095) which has much of its hydroxymethylcytosine replaced by cytosine (J. S. Wiberg, personal communication) suffer 50-70% killing under these conditions (R. Simmons and E. C. Friedberg, unpublished observations).

THE BIOLOGICAL DISTRIBUTION OF URA-DNA GLYCOSYLASE

A number of studies have reported the presence of ura-DNA glycosylase in extracts of mammalian cells and tissues (15, 40, 41). We have detected the activity in both nuclear and mitochondrial-containing fractions of human KB cells grown in suspension culture (C. Anderson and E. C. Friedberg, unpublished observations). In view of its demonstrated role in the excision of uracil during DNA synthesis in *E. coli* (36) and in human lymphocytes (M. Goulian, personal communication), it might be anticipated that this enzyme is associated with all living forms. However in recent studies using extracts of a number of drosophila cell lines in culture, as well as extracts of drosophila embryos, larvae, pupae and adult flies, prepared

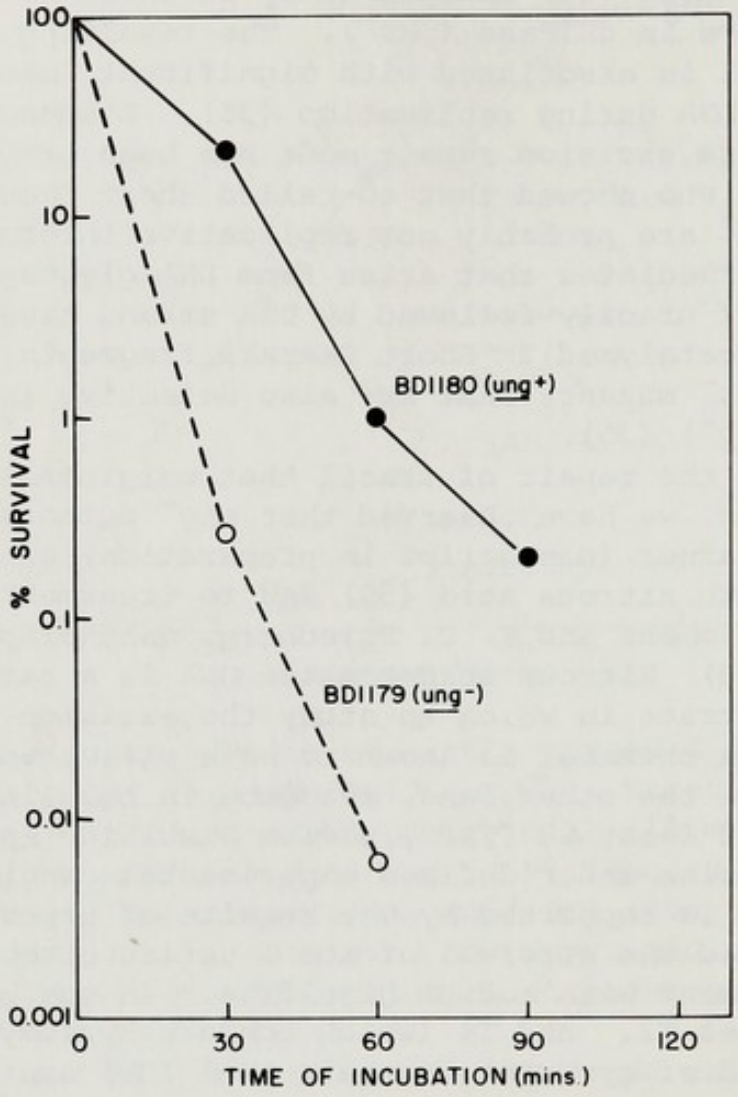

FIGURE 3. Relative sensitivity of *E. coli ung*$^+$ and *ung*$^-$ strains in stationary phase to treatment with sodium bisulfite (0.9 M). See R. Simmons and E. C. Friedberg (manuscript in preparation) for further experimental details.

by a variety of procedures, we have been unable to demonstrate this activity (R. Cone, J. B. Boyd and E. C. Friedberg, unpublished observations) using [^{3}H] poly (dU) as a substrate. The same result holds true in preparations freed of nucleic acid. In addition, mixing drosophila extracts with extracts of *B. subtilis* does not inhibit the activity in the latter. While it remains possible that our failure to detect this enzyme in drosophila preparations is an experimental artifact, it is also possible that these results have biological relevance. Drosophila flies and cells in culture grow at optimal temperatures between about 18-25°C. Duncan and Weiss (this volume) have observed a 15-fold increase in the frequency of spontaneous G:C → A:T transition mutations in *ung*$^-$ mutants of *E. coli*, presumably reflecting the rate of spontaneous cytosine deamina-

tion at 37°C. However , perhaps in organisms growing at 20-25°C the rate of cytosine deamination is not significant as a mutator effect. Using rate constants for cytosine deamination in *E. coli* DNA at 70°C and 80°C quoted by Lindahl and Nyberg (42), we calculated the rate constants at 37°C and 20°C by extrapolation on an Arrhenius plot and obtained a value for the latter temperature which is 20 fold lower than at 37°C. Thus the absence of ura-DNA glycosylase in drosophila is not inconsistant with the suggestion of Duncan and Weiss that the enzyme evolved primarily for the repair of uracil arising from cytosine deamination, rather than for the repair of uracil incorporated during DNA synthesis. However, further studies are required to establish this interesting biological distinction more clearly.

THE MOLECULAR MECHANISM OF BASE EXCISION REPAIR

A number of workers (15, 31) have suggested models for a degradative repair pathway of base excision repair that involves endonucleolytic cleavage of a phosphodiester bond at sites of base loss, presumably catalyzed by one or more of the apurinic endonucleases previously mentioned. As indicated above, mutants defective in endonuclease VI (exonuclease III) are abnormally sensitive to methylmethanesulfonate and to nitrous acid. Gossard and Verly (31) have reported that this enzyme catalyzes cleavage of a phosphodiester bond on the 5' side of a site of base loss, leaving 3'OH and 5'P termini. The deoxyribose-phosphate residue could thus be excised by a 5' → 3' exonuclease activity such as DNA polymerase I (43), and repair synthesis and rejoining could occur as in the classical model of nucleotide excision repair (1-5). Since the detailed mechanism of action of other apurinic endonucleases has not been reported, it is possible that alternative modes exist for excising sugar-phosphate residues from DNA. Finally, it is at least theoretically plausable that base excision could be completed by a non-degradative pathway involving the direct transfer of an appropriate base to the site of base loss in DNA.

ACKNOWLEDGMENTS

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WORKSHOP SUMMARY:
ENZYMOLOGY OF BASE EXCISION REPAIR

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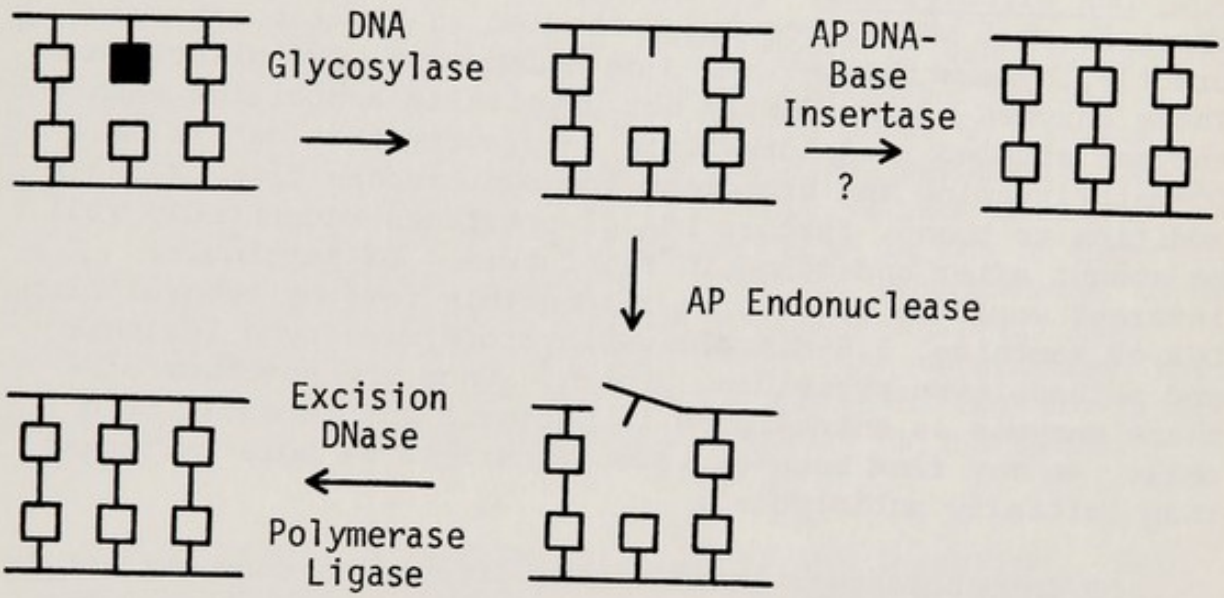
The concept of base excision repair is only several years old, yet enzymological studies have progressed sufficiently so that we might define the following pathway (or pathways):



The session focused mainly on enzyme properties, and the accompanying reports should be consulted for particular details and references.

BIOLOGICAL SIGNIFICANCE

Weiss presented evidence for the involvement in *E. coli* of uracil-DNA glycosylase and the AP endonuclease activity associated with exonuclease III (endonuclease VI) in the removal of uracil from DNA. Thus, *dut* (dUTPase) mutants incorporate uracil into DNA, but are incompatible with a simultaneous presence of the *xth* mutation, presumably because of the involvement of the endonuclease VI activity of the *xth* gene product in the removal of apyrimidinic lesions. However,

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viability is restored by the presence of the third, *ung* (uracil - DNA glycosylase) mutation. Taken together these data implicate the endonuclease VI activity and the glycosylase in the major pathway for DNA - uracil excision.

Lindahl also summarized evidence for the involvement of uracil - DNA glycosylase in uracil excision. In addition he described the *tag* mutants of *E. coli* which were deficient in 3-methyladenine - DNA glycosylase. These mutants cannot excise 3-methyladenine formed *in vivo*, identifying the glycosylase as the primary enzyme for the removal of this residue from *E. coli* DNA.

In summary, it has become clear that base-excision repair is a major component for maintaining DNA integrity.

ENZYME STUDIES

DNA Glycosylases. Lindahl summarized his data on the three *E. coli* DNA glycosylases studied in his laboratory: uracil-, hypoxanthine-, and 3-methyladenine - DNA glycosylases. These enzymes are evidently not physically associated with the enzymes studied in Goldthwait's laboratory that can release O^6-methylguanine and bromomethylbenzanthracene from DNA. In addition to these, further DNA glycosylases undoubtedly will be sought after and found in the future. Of particular interest would be activities responsible for the removal from DNA of xanthine, 5,6-dihydroxy-dihydrothymine-type lesions, and perhaps even pyrimidine dimers. Once the spectrum of these enzymes is established in bacteria and in eucaryotic cells, we may find base-excision repair to be more important than initially anticipated.

AP Endonucleases. Most of the discussion concentrated on the *E. coli* enzymes. Goldthwait summarized studies from his laboratory, stressing the necessity for obtaining a definitive classification of these enzymes, particularly with regard to their association with DNA glycosylase and exonuclease activities. Verly summarized his studies of the AP endonuclease activity, *E. coli* endonuclease VI. He stressed the fact that the use of apurinic DNA as substrate yields a different enzyme from that obtained with alkylated DNA substrates. He also noted the ubiquity of AP endonucleases in both lower and higher organisms.

Weiss presented genetic and biochemical evidence for the association in one protein of four activities: DNA phosphatase, exonuclease III, endonuclease VI, and RNase H. The cleavage site of the endonuclease (5' to the base-free sugar) is not easy to reconcile with the 3' → 5' polarity of the exonuclease. Weiss suggested a resolution of this dilemma by proposing common substrate recognition features for each of

the enzyme activities, whereas Verly suggested the utility of exonuclease III in preventing resealing of the AP endonuclease product by DNA ligase. In the latter regard the sealing of nicked AP sites by ligase has yet to be demonstrated.

Turning to human cells, Linn described studies of AP endonuclease in human placenta and fibroblasts. Whereas cultured xeroderma pigmentosum D group fibroblasts appeared to lack a major AP endonuclease activity, Linn cautioned that it would be premature to associate this defect with disease symptoms. Particularly curious in this regard was the apparent better-than-normal proficiency of these cells in the reactivation of transfecting depurinated SV40 DNA.

Within the next several years AP endonucleases should be well-described regarding their associated activities and incision details. Such information will then allow the identification of the companion excision enzymes.

Excision and Resynthesis. This action is usually expressed as a sequence: (1) excision exonuclease, (2) DNA polymerase, (3) ligase. However, several words of caution should be noted. We do not know whether polymerization is subsequent to excision, or proceeds it *via* strand displacement. We also do not know whether excision is *via* an exonuclease with a defined polarity, a bipolar exonuclease, or an endonuclease recognizing distorted or displaced DNA. Conceivably the choice of the excision mode will depend upon the particular AP endonuclease utilized. Hence the enzymic study of excision must follow mechanistic studies of the incision event.

AP Binding Proteins and Purine - AP DNA Insertion. Linn discussed the preliminary characterization of a protein from cultured human fibroblasts of 120,000 daltons that specifically binds to apurinic DNA. The preparation has the further property of apparently replacing purines, but not methylated purines at apurinic sites, putting A (not G) into depurinated poly (dA-dT) and G (not A) into depurinated poly (dG-dC). The insertion activity is inhibited by caffeine. Of particular interest will be future studies on the question of whether this protein -- or other similar proteins -- will transfer pyrimidines at depyrimidinated sites.

Should this activity be part of an alternate base excision repair pathway, the fidelity of this replacement compared to that by DNA polymerase is of interest. Alternatively, one might imagine the utility of a base - insertion mechanism not for DNA repair, but for situations where the generation of genetic diversity is desired, or where naturally occurring, modified bases are to be replaced by normal ones as a regulatory or developmental signal.

Miscellaneous factors. Two other areas for future research in base-excision repair are (1) the possible demonstration and isolation of repair complexes, capable of carrying out multistep processes, and (2) the isolation of specific binding proteins whose function might be to identify damaged regions in naked DNA or in chromatin.

APPENDIX - SOME POINTS ON NOMENCLATURE

Several topics were discussed in order to recommend standardized nomenclature for base-excision repair.

AP Endonuclease. More formally, "endodeoxyribonuclease AP," this designation should refer to an enzyme acting at both apurinic and apyrimidinic sites in DNA. Likewise, "AP DNA" will not distinguish which type of lesion is present. "Apurinic endonuclease" and "apyrimidinic endonuclease" will be reserved for enzymes specific for one or the other lesion in the event that such an enzyme should be described.

DNA Glycosylase. "Glycosylase" will replace "glycosidase." For example, "uracil-DNA glycosylase."

E. coli AP endonucleases. There is already much inconsistency in the literature, but hopefully future confusion can be avoided. Thus it is proposed that "*E. coli* endonuclease II" refer to an activity degrading alkylated DNA. At this point it is not established whether this activity is due to one enzyme or to a combination of separable glycosylase and AP endonuclease proteins. "*E. coli* endonuclease VI" should refer to the activity specific for AP sites isolated by Verly and Rassart in 1975. It is likely to be equivalent to the AP endonucleolytic activity of exonuclease III.

DNA GLYCOSYLASES OF ESCHERICHIA COLI[1]

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ABSTRACT E. coli contain several different enzymes which catalyze the hydrolytic release of unusual or damaged bases from DNA, with the consequent formation of apurinic or apyrimidinic sites. These enzymes are of relatively low molecular weight and do not require cofactors. Each enzyme is highly specific for a particular kind of lesion. Thus, one enzyme releases uracil, a second hypoxanthine, and a third 3-methyladenine and 3-ethyladenine from DNA.

INTRODUCTION

Enzymes that act in the nucleoside catabolism of yeast and Lactobacillus by catalyzing the hydrolysis of uridine to uracil and ribose, or inosine to hypoxanthine and ribose, have been known for many years (1, 2). Recently, enzymes with a similar mode of action on DNA were discovered (3-6). They were initially referred to as N-glycosidases but are now called DNA glycosylases. The main reason that the existence of DNA glycosylases was overlooked for so long is no doubt the narrow specificity of this group of enzymes for certain types of unusual or damaged nucleotide residues in DNA. No DNA glycosylases liberating the commonly occurring bases under any conditions have so far been detected. E. coli contain at least three different enzymes of this class, which act on deaminated or alkylated DNA. The substrate specificity of these enzymes initially suggested that they were active in DNA excision-repair, and this notion has now been confirmed by the isolation and characterization of enzyme-defective E. coli mutants (7, 8).

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RESULTS AND DISCUSSION

Uracil-DNA glycosylase. The E. coli enzyme has been purified to homogeneity (9). The native enzyme is a monomer of molecular weight 24 500. It does not require Mg^{2+}, P_i, or other cofactors for activity, and it efficiently releases free uracil from single-stranded DNA and from double-stranded DNA containing either adenine-uracil or guanine-uracil base-pairs. On the other hand, it does not liberate 5-methyluracil, 5-bromouracil, hypoxanthine, or alkylated bases from DNA, nor does it cleave free deoxyuridine, dUMP, or UMP residues in RNA. Chain breaks are not directly introduced as a consequence of the release of uracil from DNA, but the DNA becomes sensitized to endonucleases acting at apurinic/apyrimidinic sites. The enzyme is product-inhibited. Uracil-DNA glycosylase may thus be selectively inhibited in crude lysates or permeabilized cells by addition of free uracil (10).

E. coli mutants, ung, defective in uracil-DNA glycosylase have been isolated by a mass screening procedure (7). However, in our experience none of the mutants isolated to date seems to be completely deficient in enzyme activity. The tightest mutants differ from wild type strains in that they are nitrous acid-sensitive (11), have an increased spontaneous mutation frequency (7), and do not effectively remove misincorporated dUMP residues from DNA (12).

Hypoxanthine-DNA glycosylase. Hydrolytic, nonenzymatic deamination of adenine to hypoxanthine in DNA, albeit considerably slower than the deamination of cytosine residues, still occurs at a rate that appears to be of physiological significance (13). By analogy to uracil-DNA glycosylase, it seemed possible that an enzyme with the ability to release hypoxanthine from DNA might exist, and we have recently demonstrated the presence of such an activity in E. coli extracts (14). Hypoxanthine-DNA glycosylase activity is present at a much lower level than uracil-DNA glycosylase activity in crude cell extracts. Further, hypoxanthine-DNA glycosylase shows different fractionation properties than either uracil-DNA glycosylase or 3-methyladenine-DNA glycosylase. The partly purified enzyme shows a preference for double-stranded substrates, and releases hypoxanthine 10 times faster from poly (dA, [^{3}H]dI) poly (dT) than from single-stranded poly (dA, [^{3}H]dI). Hypoxanthine is also released from nitrous acid-treated DNA by this enzyme without simultaneous liberation of adenine or guanine. On the other hand, purine bases with other alterations in the 6 position, O^6-methylguanine and 6-methyladenine, are not removed from DNA.

Since E. coli cells do not phosphorylate IMP to IDP (15), it is presently unclear if misincorporation of dIMP residues into DNA occurs to any significant extent, or if the enzyme only serves to remove deaminated dAMP residues from DNA.

3-Methyladenine-DNA glycosylase. The E. coli enzyme has recently been extensively purified and characterized (16). A similar enzyme is also present in Micrococcus luteus (17). E. coli 3-methyladenine-DNA glycosylase releases 3-methyladenine and 3-ethyladenine, but not 7-methylguanine, 7-ethylguanine, O^6-methylguanine, O^6-ethylguanine, N^6-methyladenine, or the bulky adducts introduced by bromomethylbenzanthracene treatment from double-stranded alkylated DNA. The native enzyme has a molecular weight of 19 000 and does not introduce chain breaks into alkylated, X-irradiated, or depurinated DNA, so it is clearly different from the preparations of endonuclease II investigated by Goldthwait's group (6).

E. coli mutants deficient in 3-methyladenine-DNA glycosylase, tag, have recently been isolated as methyl methanesulfonate-sensitive mutants (8). They show normal resistance to ultraviolet or ionizing radiation. Our tightest tag mutant has been shown to have lost the ability to excise actively 3-methyladenine from DNA alkylated in vivo. On the other hand, the ability to release O^6-methylguanine has been retained, in accord with the specificity of the purified enzyme. These studies (a) identify 3-methyladenine as a lesion having a major contribution to lethality in methyl methanesulfonate-treated E. coli in the apparent absence of a repair enzyme, (b) identify 3-methyladenine-DNA glycosylase as the enzyme responsible for active removal of 3-methyladenine from the DNA of E. coli cells treated with alkylating agents.

Additional DNA glycosylases. Two of the three DNA glycosylases described here act on lesions that may occur as a consequence of nonenzymatic hydrolytic deamination of DNA at moderate temperatures, and additional enzymes of this class may well exist to initiate repair of other types of spontaneous DNA lesions. One obvious example of such a lesion would be xanthine (deaminated guanine). However, it should be noted that xanthine, in contrast to uracil and hypoxanthine, may not induce mispairing so a repair system to handle this lesion may not be essential. The physiological role of 3-methyladenine-DNA glycosylase is presently less clear, but may be resolved with the aid of the recently isolated E. coli tag mutants. While this enzyme clearly is active in the repair of a lesion introduced by many types of alkylating agents, it seems doubtful that

living cells have been so frequently exposed to alkylating agents during evolution that it became necessary to develop a separate pathway of DNA repair. Instead, the possibility that enzymatic methylation of DNA adenine residues in the 3 position may occur under in vivo conditions should now be seriously considered.

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URACIL-DNA GLYCOSYLASE MUTANTS ARE MUTATORS[1]

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ABSTRACT Spontaneous mutation to nalidixic acid or to rifampicin resistance is increased 5-fold in uracil-DNA glycosylase (ung) mutants of E. coli. By trpA reversion analysis we observed a 15-fold increase in the frequency of spontaneous G:C→A:T transition mutations while other transition and transversion frequencies were not affected. Duncan and Warner (Nature, 1978) have previously found that uracil-containing phage (30% uracil) are viable in ung⁻ bacteria; the uracil is not removed from the DNA. Thus, uracil-DNA glycosylase appears to have evolved not to prevent uracil misincorporation into DNA (as A:U), but rather to edit (mutagenic) deaminated cytosine residues. This mechanism precludes the use of uracil as a "normal" DNA constituent.

INTRODUCTION

Uracil has not been considered a normal component of DNA. Mutagens such as bisulfite or nitrous acid, however, were thought to produce uracil in DNA by the deamination of cytosine. The resulting stable G:U "base-pairs" would then lead to G:C→A:T transitions after the next round of replication. With the discovery that spontaneous deamination of cytosine residues occured at significant rates came the realization that there might be cellular repair systems for the removal of uracil from DNA (1). Two E. coli enzymes active on uracil-containing DNA, endodeoxyribonuclease V (2) and uracil-DNA glycosylase (3), were subsequently characterized, and glycosylase-deficient (ung) mutant strains were isolated.

Uracil-DNA glycosylase specifically removes uracil that arises from the deamination of cytosine in DNA, or that has been misincorporated into DNA in place of thymine (3,4). Studies of ung, dut (dUTPase⁻), and xth (exonuclease III⁻)

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TABLE I
EFFECT OF *ung* ON SPONTANEOUS MUTATIONS TO ANTIBIOTIC RESISTANCE

Strain	Antibiotic	Mutants per[a] 10^7 cells	Enhancement *ung*$^-$/*ung*$^+$
KL16	Rif	6.1	(1.0)
KL16 dut-1	Rif	12.3	2.0
KL16 ung-1	Rif	24.9	4.1
KL16 dut-1 ung-1	Rif	34.0	5.6
BD1154	Nal	2.1	(1.0)
BD1154 ung-1	Nal	11.0	5.4
BD1154	Rif	1.5	(1.0)
BD1154 ung-1	Rif	6.9	4.5

[a]Frequencies were determined as a number average after marked fluctuations were discarded (9).

have also resulted in the following observations: (a) *dut* mutants incorporate uracil into DNA and its repair leads to the fragmentation of nascent DNA (4); (b) this fragmentation is prevented by the *ung* mutation (5); (c) uracil is stably incorporated into cellular and viral DNA in mutant *dut ung* bacteria (5,6); (d) such uracil-containing DNA coliphages will grow in *ung*$^-$ but not *ung*$^+$ bacteria (6); and (e) the transduction of an *ung* mutation into *dut xth* double mutants (inviable at 37°) restores their viability (7). These results indicate that uracil-DNA glycosylase is a key enzyme in preventing the persistence of uracil in DNA, but the fact that uracil-containing bacteria and phage survive suggests that the enzyme did not evolve to prevent misincorporation of uracil in place of thymine. We have recently found that *ung* is also a weak mutator; we present results suggesting that uracil-DNA glycosylase functions as an editing (or anti-mutator) enzyme to repair deaminated cytosine residues in DNA.

RESULTS AND DISCUSSION

Mutator Effect of ung. Table I shows that the frequencies of spontaneous mutation to nalidixic acid resistance (Nal^R) and to rifampicin resistance (Rif^R) were increased in two different *ung*$^-$ strains as compared to those of their congenic *ung*$^+$ counterparts. If the mutator effect of *ung* mutations is due to the persistence of uracil-thymine substitutions (A:U base-pairs in DNA) then the addition of a *dut*

TABLE II
BASE-PAIR SPECIFICITY OF *ung* MUTATOR EFFECT

trpA allele	Reversion Pathway	Revertants/10^{10} cells[a] ung$^+$	ung$^-$	Enhancement ung$^-$/ung$^+$
23	A:T→G:C, C:G	46	37	0.8
58	A:T→G:C	56	51	0.9
78	A:T→C:G	130	210	1.6
223	A:T→G:C, C:G	32	42	1.3
446	G:C→A:T	7	100	14.7

[a]Reversion frequencies were determined using a modified fluctuation test (9). Thirty cultures were used to determine number averages. Medians were approximately equal to the means.

mutation, which increases such substitutions, should have enhanced the mutation rates. Because it did not (Table I) and because there is very little dUTP incorporation into DNA in *dut*$^+$ *ung*$^-$ cells (Duncan and Warner, unpublished observation), it seems that the mutagenicity of *ung* mutations is not due to the persistence of A:U base pairs in DNA.

Specific Transitions Induced by ung. To examine the specificity of *ung* induced mutations, we studied *trpA* mutations that revert to Trp$^+$ by known base-pair substitutions (8). The spontaneous reversion frequencies of several *trpA* alleles in otherwise congenic strains showed no enhancement by the *ung* mutation (Table II). There was, however, a 15-fold increase in reversion of the *trpA446* allele in *ung*$^-$ strains. Standard testing (8) showed the revertants were true wild type, indicating that G:C→A:T transitions had occurred. The *trp* alleles for which no enhanced reversion was found were those that revert by other transition and transversion pathways (Table II).

The specific, *ung*-dependent increase in the frequency of G:C→A:T transition mutations is consistent with the idea that cytosine deamination occurs spontaneously *in vivo* and that uracil-DNA glycosylase repairs the resulting uracil-containing DNA (1,3). Thus G:U base-pairs arising from G:C are repaired before they can be converted to A:U pairs in the next round of replication. The enzyme does not distinguish between (mutagenic) G:U and (normal) A:U base-pairs. Therefore, if the glycosylase is to function as an anti-mutator enzyme, uracil cannot be a normal constituent of DNA,

and for this reason its incorporation is largely prevented by dUTPase.

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ENDONUCLEASES SPECIFIC FOR APURINIC SITES IN DNA.

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ABSTRACT : Endonucleases specific for apurinic sites have been purified from bacteria, animals and plants. In eucaryotic cells, the enzyme is present in the non-histones of chromatin. The details of the *in vitro* repair of depurinated DNA by *E.coli* endonuclease VI, exonuclease III, DNA polymerase I and ligase, have been established experimentally.

In 1968, Strauss and Robbins (1) found, in a crude extract of *M.lysodeikticus*, an endonucleolytic activity on MMS-methylated DNA; the activity was not suppressed by heat-induced depurination and the authors suggested that separate enzymes might attack at apurinic sites and at sites of the methylated purines.

The work was resumed with *Escherichia coli* by two groups which used different substrates to purify the enzymes and came to what appeared as conflicting results : Goldthwait and his colleagues used MMS-alkylated DNA, whereas Verly and Paquette, who were more interested in apurinic sites, used alkylated-depurinated DNA. The two methods yielded different preparations : Verly *et al.* (2,3,4) purified an enzyme specific for apurinic sites with no action on alkylated sites, whereas Friedberg and Goldthwait (5) obtained a preparation active on alkylated bases which they named endonuclease II. This preparation was later found to be active on apurinic sites and Hadi and Goldthwait (6) thought mistakenly that the same enzyme, endonuclease II, was active on both substrates. This was the situation at the Squaw Valley meeting on the Molecular Mechanisms for Repair of DNA held in February 1974. Kirtikar and Goldthwait (7) then made the important discovery of a N-glycosidase activity in their endonuclease II preparation. In the mean-time, Verly and Rassart (8) had completely purified, from *E.coli*, the main endonuclease specific for apurinic sites. Kirtikar and Goldthwait (9) subsequently realized that their endonuclease II preparation was a mixture of enzymes; they were able to separate the N-glycosidase, for

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which they kept the name of endonuclease II because it still had some endonuclease activity, from an endonuclease specific for apurinic sites.

The confusion, arising from the work of Goldthwait's group, led many people to name endonuclease II the enzyme acting on apurinic sites. To stop this misnomer, we finally gave the name of endonuclease VI to the enzyme we had been studying since the late sixties (10).

Escherichia coli endonuclease VI is thus the first endonuclease specific for apurinic sites which has been described (2,3); it has no action on normal DNA strands or on alkylated sites (4). The purified enzyme was found to be a monomeric protein of 32,000 daltons (8) which needs Mg^{++} to be active and is rather thermolabile. The enzyme hydrolyzes a phosphoester bond on the 5' side of the apurinic site (which might be not immediate neighbour of the base-free deoxyribose) leaving a 3'-OH and a 5'-phosphate (10,11).

According to Ljungquist et al. (12), endonuclease VI is responsible for only 90 % of the activity on depurinated DNA in E.coli; the remaining 10 % are due to a second enzyme, endonuclease IV, which is stable at 45°C and does not need Mg^{++}.

Weiss (13) published that endonuclease II is exonuclease III; what he should have written is not endonuclease II but possibly endonuclease VI. We indeed found exonuclease III in a preparation of endonuclease VI which was devoid of endonuclease II activity (10,11). Ljungquist et al. (12) found bacterial mutants in which exonuclease III and endonuclease VI had disappeared simultaneously. However, Kirtikar et al. (14) have separated exonuclease III from an endonuclease specific for apurinic sites which they present as if it were our endonuclease VI (and to which they give the name of endonuclease VI), but which Ljungquist and Lindahl (15) think to be rather endonuclease IV. The question whether exonuclease III and endonuclease VI are the same enzyme is thus not yet settled and it is much better to keep separate names for the two activities. Should they finally be recognized by everyone as the same enzyme, the name of endonuclease VI should be retained preferentially to exonuclease III : exonuclease III has been waiting many years for a biological function whereas endonuclease VI is most important for the repair of apurinic and apyrimidinic sites in DNA and has been, moreover, the first enzyme of its class to be discovered. We shall see later that the biological action of exonuclease III is likely quite accessory and, indeed, not absolutely necessary.

Depurinated DNA has been repaired in vitro by incubation with endonuclease VI, DNA polymerase I and the 4 dNTP, ligase

and its coenzyme (16); the endonuclease VI preparation contained exonuclease III. The details of the repair have been worked out (10,12) : endonuclease VI hydrolyzes a phosphoester bond on the 5' side of the apurinic site leaving a 3'-OH and a 5'-phosphate; exonuclease III removes a few nucleotides in the 3'-5' direction leaving the apurinic site in the DNA molecule (which prevents ligase from closing immediately the nick); DNA polymerase I, starting from the 3'-OH, fills the gap due to exonuclease III, excises the apurinic site in a di- or trinucleotide, then catalyzes the translation of a nick in the 5'-3' direction; the nick is closed by ligase. The antiligase activity of exonuclease III is apparently not absolutely required since apurinic sites are repaired by endonuclease IV in absence of exonuclease III (12).

Lindahl and Nyberg's paper (17) on the spontaneous depurination of DNA led us to suggest (18) that an endonuclease specific for apurinic sites must be present in every cell. We have isolated an endonuclease for apurinic sites from Bacillus stearothermophilus (19); its properties are closer to those of E.coli endonuclease IV than endonuclease VI. An endonuclease specific for apurinic sites was also purified from rat liver (20,21) and plant embryos (22). An endonuclease for apurinic sites was detected in all the rat tissues that were tested, in human fibroblasts, HeLa cells, in roots and leaves of higher plants, in fungi and algae (23). Other authors have also purified endonucleases for apurinic sites from bacteria, plant or mammalian tissues.

In eukaryotic cells, the endonuclease specific for apurinic sites is mostly located in chromatin. It is a non-histone protein which, when integrated in the chromatin architecture, has little activity on a foreign DNA; this suggests an orientation to work on chromatin DNA. These observations, first made in plant tissues (22,23), have since been confirmed using rat liver.

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THE ENDONUCLEASE ACTIVITY OF EXONUCLEASE III AND THE REPAIR OF URACIL-CONTAINING DNA IN *ESCHERICHIA COLI*[1]

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ABSTRACT In addition to its well-known exodeoxyribonuclease and DNA-3'-phosphatase activities, exonuclease III of *E. coli* has RNase H activity and is an endonuclease for apurinic/apyrimidinic sites in DNA. This endonucleolytic activity produces base-free deoxyribose-5-phosphate end groups, as predicted by a model in which all of the enzymatic activities of exonuclease III are catalyzed by a single active site. dUTPase (*dut*) mutants generate apyrimidinic sites through the misincorporation of uracil into DNA, followed by its removal. They do not tolerate exonuclease III (*xth*) mutations; *dut xth* double mutants are conditionally inviable, suggesting that exonuclease III functions in the repair of uracil-containing DNA.

INTRODUCTION

Exonuclease III of *E. coli* attacks the 3'-ends of duplex DNA to release terminal phosphomonoesters and 5'-mononucleotides (1,2). It is also an endonuclease for apurinic/apyrimidinic sites in DNA. This discovery was made during the study of mutants that were deficient in "endonuclease II" activity (3). At that time, endonuclease II activity was defined by an assay that measured the release of fragments from methyl methanesulfonate-treated DNA (4). Although the substrate contained a variety of lesions, we presumed from the work of Dr. Verly's lab (5) that the cleavage occurred primarily at apurinic sites. Our three "endonuclease II" mutants, plus one described by Ljungquist *et al.* (6), were found to be deficient in exonuclease III activity, and all 8 of our exonuclease III mutants were found to have reduced endonuclease II activities. Mutants that were temperature-sensitive for one activity were temperature-sensitive for the other. A

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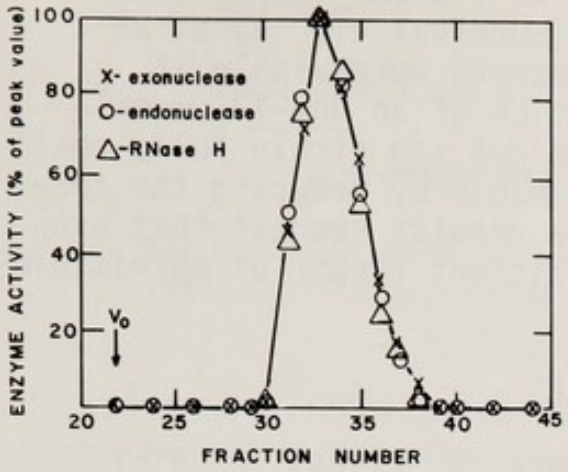
thermolabile mutant endonuclease II activity was purified by the standard endonuclease purification scheme as further proof of its identity. All mutations were at 38 min on the *E. coli* genetic map (7), and the enzymatic defects were not only co-mutable, but they were also co-revertable and co-transducible. In separate studies, we found that *Hemophilus influenzae* has a similar enzyme that possesses co-mutable exonucleolytic and endonucleolytic activities (8). Finally, homogeneous *E. coli* exonuclease III had an endonuclease II activity that was physically inseparable from it (9).

Three discoveries in Dr. Goldthwait's lab appeared to contradict our results (10,11). In their hands, (i) purified exonuclease III lacked endonucleolytic activity, (ii) an endonuclease for apurinic sites could be separated from exonuclease III, and (iii) some of our exonuclease III mutants still possessed that endonuclease activity. They then redefined endonuclease II in terms of a different assay and purification scheme, and it now refers to an enzyme with a specificity for alkylated residues in DNA (10). We attempted to resolve some of these problems.

RESULTS AND DISCUSSION

Endonuclease Activity. We repeated many of our previous experiments, but this time we used a highly specific endonuclease assay (8) that measured the nicking of partially depurinated (acid-treated) supercoiled molecules of ØX174 RFI DNA. We confirmed that exonuclease III has an endonuclease activity specific for partially depurinated (versus non-depurinated) DNA. We invariably found that the two activities were co-mutated, co-reverted, co-transduced, and co-purified; one such experiment is shown in Fig. 1. We also found that the endonuclease activity of exonuclease III was inhibited 75-fold under the conditions of Kirtikar *et al.* (10,11) (*i.e.*, 0.1 mM 8-hydroxyquinoline and no Mg^{2+}), perhaps accounting for their failure to observe that activity. Their DNase for apurinic sites, therefore, must be another enzyme because it was separated from exonuclease III and it was present in some exonuclease III mutants (11).

Cleavage Site. Exonuclease III is a monomeric protein (M_r-28,000). Its small size suggested that one active site catalyzes all of its enzymatic reactions. The common-site model (9) predicted that the enzyme should cleave on the 5'-side of a base-free sugar-phosphate, just as it cleaves on the 5'-side of a terminal nucleotide or of a terminal phosphate. To test this hypothesis, we synthesized $d(A\text{-}T)_n$ containing occasional 5'-[^{32}P]dUMP residues (8) and treated



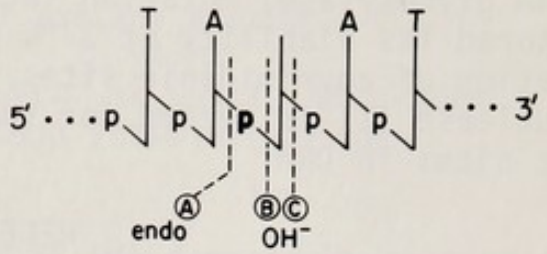


FIGURE 1 (Left). Sephadex G100 chromatography of purified exonuclease III (8). Assay methods: endonuclease for apurinic sites (11); RNase H measured like the exonuclease (8) but with 2.4 μM [^{3}H]poly(A)·poly(dT) as substrate.

FIGURE 2 (Right). Site of endonucleolytic cleavage. ^{32}P on the 5'-side of the apyrimidinic site became susceptible to release by phosphatase or alkali. See text.

it with uracil-DNA glycosylase followed by exonuclease III. When 2-5% of the DNA became acid-soluble, 21% of the ^{32}P could be released into a Norit-nonadsorbable form either by alkaline phosphatase or by NaOH (Fig. 2). These results confirmed our prediction.

RNase H Activity. Exonuclease III can degrade DNA strands containing ribonucleotides (1,12). We found that the purified enzyme degraded RNA strands, but not DNA strands, in a DNA-RNA hybrid. This RNase H activity (Fig. 1) suggests that the enzyme has a recognition site for a deoxyribose on the strand opposite the one it cleaves, thereby also explaining its specificity for duplex substrates.

Biological Role. Exonuclease III is expendable. A deletion mutant (7) appears to be healthy and to contain 10-15% of wild type endonucleolytic activity for apurinic sites. We studied the biological role of exonuclease III by combining the deletion mutation (Δ*xth*) with a *dut* mutation. *dut* (dUTPase) mutants incorporate uracil into DNA, and its removal by uracil-DNA glycosylase should generate apyrimidinic sites. The *dut-1* mutant has a prolonged generation time and displays excessive fragmentation of nascent DNA, due presumably to the rapid incision of apyrimidinic sites by

repair endonucleases (13-15). At 37°, a dut-1 Δxth double mutant formed filaments and died (survival $<10^{-4}$); at 25°, it survived. dut or xth single mutants were viable at either temperature. The transduction of an ung-1 (uracil-DNA glycosylase) mutation into the dut xth strain fully restored its viability at 37°, presumably by reducing the formation of apyrimidinic sites. The results suggest that exonuclease III is needed for the efficient repair of apyrimidinic sites in DNA.

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BASE-EXCISION REPAIR IN MICROCOCCUS LUTEUS[1]

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ABSTRACT Base-excision repair of DNA damaged by chemicals relies upon the sequential action of specific DNA-glycosylases wich excises the modified bases yielding an apurinic or apyrimidinic site. This site is in turn recognised by a specific endonuclease wich hydrolyse the phosphodiester bond of DNA adjacent to this site. In M.luteus an uracil-DNA-glycosylase split off uracil in a DNA containing such a base. An independant 3-methyladenine DNA-glycosylase liberates 3-methyladenine from alkylated DNA but not 7-methylguanine. The apurinic or apyrimidinic sites are recognised by one of the two endonucleases specific for such sites and hydrolyse the phosphodiester bond of DNA.

INTRODUCTION

Some chemicals react with bases in DNA yielding non conventional or modified bases. Among them, alkylating agents yield alkylpurine mostly 7-methylguanine (7-MeGua), 3-methyladenine (3-MeAde) O^6-methylguanine (1). Nitrous acid induces cytosine, adenine and guanine deamination yielding respectively uracil, hypoxanthine and xanthine. Some of this modified bases are actively repaired in bacteria through "base-excision repair" a term proposed by Friedberg (2) to emphasize the release of the damaged residue as a free base.Such a mechanism was hypothesised by Lindahl (3) and first demonstrated to occur in M.luteus in the repair of alkylated bases (4,5) and then in E.coli for uracil containing DNA (6).This mechanism, for the incision of the DNA backbone involves the sequential action of a specific DNA-glycosylase which liberates the damaged bases yielding an apurinic or apyrimidinic site (AP-site) which is in turn recognised by an endonuclease specific for such sites. We report here the purification and characterization of the uracil DNA-glycosylase and a new AP-endonuclease in addition to the 3-MeAde-DNA-glycosylase and the AP-endonuclease already described (5).

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DNA-GLYCOSYLASES

So far, two DNA glycosylases have been purified and characterized in M.luteus

3-MeAde-DNA-Glycosylase. The assay measures ethanol soluble products liberated from (^{3}H) dimethyl sulfate alkylated DNA (DMS-DNA).Purification procedure involves chromatography on DEAE-cellulose, P-cellulose and DNA-Agarose (5).The enzyme has no unspecific endonucleolytic activity (native supercoiled [^{3}H] PM_2-DNA), no AP-endonucleolytic activity (supercoiled [^{3}H] PM_2-DNA heat depurinated), no exonucleolytic activity (native or heat denaturated [^{14}C] E.coli DNA) no uracil-glycosylase (see below). It liberates 3-MeAde from DMS-DNA but no 7-MeGua.

Uracil-DNA-Glycosylase. The assay measures acid soluble products liberated from the uracil containing DNA of the phage PBS 1 (6). Purification procedure includes phase partition (7) of the lysate,DEAE-cellulose, P-cellulose, hydroxyapatite. The enzyme,thus obtained,is homogeneous as judged by acrylamide gel electrophoresis. It has no unspecific endo or exonuclease, no AP-endonuclease, no 3-MeAde-DNA-glycosylase (see above) It is specific for uracil in either double or single stranded DNA but it is twice as active on the latter. It has a molecular weight of 19,000 daltons.

ENDONUCLEASES FOR AP-SITES

The assay measures the perchloric soluble oligonucleotides liberated from E.coli [^{3}H] DNA heavily alkylated with MMS and subsequently (5). After lysis of the cells by lysozyme and sonication to reduce the viscosity, nucleic acids are removed by phase partition (7). The proteins are purified by DEAE-cellulose then P-cellulose wich separate two peaks of enzyme activity A and B. These two activities are separatelypooled and further purified by DNA-Agarose. The peak B accounted for 80% of the total activity. It is the activity already purified and described (5). Both activities are devoid of unspecific endo or exonuclease, uracil-DNA-glycosylase and 3-MeAde-DNA-glycosylase (see above). These two enzymes have comparable requirements : optimum pH being between 7.0 and 8.0, no absolute requirement for Mg^{2+} or reducing agents although they are more active in their presence. Some properties of the two enzymes are given in Table 1.

DISCUSSION

The results reported above bring some insights on the pathway of base excision repair in M.luteus. It should be emphasized that it is with enzymes isolated from this bacteria that we have been able to show for the first time (5) that strand incision of alkylated DNA is a two steps mechanism.

TABLE I
CHROMATOGRAPHIC AND PHYSICAL PROPERTIES OF AP ENDONUCLEASES

	AP-endo A	AP-endo B
Molarity of enzyme elution on		
- P-cellulose (M KCl)	0.20	0.29
- DNA-Agarose (M KCl)	0.27	0.12
- Hydroxyapatite (M PO_4^{---})	0.20	0.12
Exonuclease activity	none	none
Isoelectric point	4.8	8.8
Sedimentation coefficient (S)	2.8	2.8
Half life at 45°C (min.)	3	13

It involves the sequential action of a specific 3-MeAde-DNA-glycosylase and a AP-endonuclease. This mechanism was extended to E.coli for the repair of alkylated DNA (8) and of uracil in uracil containing DNA (6).

The base excision pathway is expected to handle the repair of other faulty bases as Lindahl et al.(8) reported a new DNA-glycosylase in E.coli which excises hypoxanthine in DNA. We have preliminary experiments with M.luteus suggesting that it could also be true for X-rays induced lesions in DNA.

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ENZYMES FROM HUMAN FIBROBLASTS FOR THE REPAIR OF AP DNA[1]

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ABSTRACT AP endonuclease activity from cultured human fibroblasts was resolved into two species by phosphocellulose chromatography with Km's for apurinic sites of 5 and 44 nM, respectively. The low Km species was not detected in extracts of Xeroderma pigmentosum group D cell lines XP5BE, XP6BE, or XP7BE. In assaying for the endonuclease activity by its ability specifically to bind apurinic DNA we resolved from the endonuclease activity a 120,000 dalton protein that also specifically binds to this DNA. The protein appears to have the ability to reinsert purines into the apurinic DNA residues.

INTRODUCTION

Our laboratory has recently reported the characterization of human AP endonuclease activities (1,2). The activity appears to resolve into several species, each of which acts on AP lesions formed by acid, alkylating agents, uracil-DNA glycosylase, X-rays or O_sO_4. The incision is made so as to form a deoxyribose 5'-phosphate and a 3'-hydroxynucleotide. In addition it was noted that AP endonuclease in extracts from some lines of xeroderma pigmentosum (XP) complementation groups A and D had reduced affinity for apurinic DNA. In this report we summarize some recent work on AP endonuclease in some XP-D lines and the characterization of a protein that binds specifically to apurinic DNA.

AP ENDONUCLEASE

When partially-purified AP endonuclease from cultured human fibroblasts was chromatographed on phosphocellulose

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approximately one-fourth of the recovered activity (assayed with saturating substrate levels) flowed through the column, whereas the remainder eluted at 240 mM potassium phosphate (3). Both fractions specifically nicked all of the apurinic sites, but they differed in their sedimentation coefficients and Km's for apurinic sites: $S^{w}_{20} = 3.3$, Km = 4.6 ± 1.3 nM apurinic sites for the flow-through species; $S^{w}_{20} = 2.8$, Km = 44 ± 17 nM for the high-salt eluate.

The same distribution of activity was noted for extracts from several other normal lines, XP-A lines, an ataxia telangiectasia line and HeLa (3). However, extracts from the XP-D lines XP5BE, XP6BE, and XP7BE lacked the flow-through activity. Since the cellular concentration of AP sites is generally quite low, the lack of this activity would be a major loss to the cell. However, until characterization proceeds to more cell lines, and until more of the details are known about the biochemistry, regulation, fidelity and relative importance of individual repair pathways, it would be premature to correlate DNA repair enzyme deficiencies with disease symptoms.

APURINIC DNA BINDING PROTEIN AND PURINE INSERTION

Isolation and Detection of Purine Insertion Activity. In order to simplify the assay for AP endonuclease, we attempted to adapt the nitrocellulose filter binding assay for UV corr-endonuclease of Braun and Grossman (4). Such an assay would circumvent interference by non-specific endonucleases that accumulate in the flow-through species. Whereas both AP endonuclease species showed specific apurinic DNA binding, sedimentation in glycerol of the high-salt eluate resulted in the appearance at 6.4S (equivalent to roughly 120,000 daltons) of a specific binding protein well-resolved from the 2.8S endonuclease. In addition the protein did not bind to freshly UV-irradiated DNA and was distinguishable from the UV-irradiated DNA binding protein described by Feldberg and Grossman (5) which had eluted earlier in the phosphocellulose column.

To determine the function of the binding protein, we tested whether it would protect DNA containing AP sites against cleavage by alkali. Whereas the protein had no effect alone, it could protect as many as one quarter the sites induced in PM2 DNA by acid treatment (1,3) when either G, GdR, dGTP, A, AdR, or dATP (but not 7-methylguanine or 6-methyladenine) were present with the protein. When both G and A were present, half of the sites were protected.

To determine whether the purine requirement reflects direct base insertion, the incorporation of radioactive label into acid-treated DNA was monitored. ^{3}H was incorporated from ^{3}H-labelled G, A, GdR, or AdR, but not from T or TdR. The incorporation was linear for several hours and was proportional

TABLE 1
FATE OF INCORPORATED GUANINE-^3H[a]

	fmol recovered as		
	G	GdR	dGMP
omit DNA	180	29	9
complete reaction	224	184	439

[a]DNA with roughly 2900 fmol depurinated sites was reacted so as to incorporate 760 fmol [^3H]guanine into acid-insoluble material. The material was collected with acid onto a glass fiber filter, washed, digested with pancreatic DNase and venom diesterase, then chromatographed upon cellulose thin layer plates. The nucleoside probably appeared because of phosphomonoesterases in the venom diesterase.

to the protein added in the early phase of the reaction. It was equally efficient with acid-treated, closed, circular PM2 phage DNA, linear T7 phage DNA or *E. coli* DNA. None was found with non-depurinated DNAs.

The incorporation of purine label with the concomitant resistance of apurinic sites to alkali, naturally suggested the conversion of these sites to normal nucleotides. To confirm this hypothesis the fate of the incorporated label was followed after incorporation into DNA and subsequent digestion of the DNA to dNMP residues (Table 1). Indeed, by this protocol, the majority of incorporated label originating in free guanine could subsequently be recovered as dGMP.

Reaction specifics. The purine insertion is optimal near pH 7.5, requires K^+ but not Mg^{++}, is inhibited by EDTA, and requires no obvious energy-related cofactor. Heating or freezing of the protein inactivates it, and caffeine inhibits A or G incorporation by 20 - 80% in the range of 0.1 - 1 mM.

If we assume that (*1*) the AP binding protein and purine-apurinic DNA insertion activity reside in the same molecule, (*2*) that each molecule is active in both reactions, and (*3*) that one molecule of binding protein per DNA molecule is seen by our binding protein assay, then we estimate the turnover number to be 400 purines inserted per protein molecule per hour.

Finally, to test whether the purine transfer has a tem-

TABLE 2
TEMPLATE SPECIFICITY

Depurinated Acceptor	fmol Base Incorporated	
	G	A
Poly (dA-dT)	< 20	231
Poly (dG-dC)	176	< 20
T7 DNA	170	—

plate specificity, depurinated Poly (dA-dT) and Poly (dG-dC) were used as acceptors (Table 2). Both polymers were as active as T7 DNA, and purine transfer appeared to be template-specific.

Conclusions. We, of course, have no evidence for the function of the purine insertase activity. However, the existence of an alternative repair pathway for apurinic DNA was suggested to us by experiments with host cell reactivation of transfecting SV40 viral DNA (R. Kdruna, S. Linn, E.E. Penhoet, unpublished). Contrary to our expectations, the XP-D lines that lack a major AP endonuclease species are *more* effective than normal in the reactivation of the depurinated viral DNA, though they are very defective in reactivation of UV-irradiated DNA. Perhaps an alternative, normally silent repair mechanism is more efficient for host cell reactivation and is exhibited in these cells.

The results presented here are clearly preliminary. In particular, an immediate question is the possible ability of this, or a separable activity to insert pyrimidines into apyrimidinic sites. Also, studies in bacteria are appropriate so that the relevant genetic experiments can be done.

A final question worthy of investigation is that of energy. We assume that the formation of a glycosidic bond requires energy, although the free energy of formation within a DNA molecule is difficult to estimate. Our only suggestion for the free energy source in the case of a free purine substrate (assuming that the enzyme does turn over) is the reforming of base pairs and stacking at the site of the reaction. Such a source would not likely be so great as triphosphate hydrolysis. If fidelity is dictated by "kinetic proofreading" (6), then we might imagine that the purine insertion pathway could be more error-prone than a pathway utilizing polymerase and triphosphate substrates. Finally, the fact that guanine is the substrate for a tRNA guanylation enzyme (7,8) provides a precedent.

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ENZYMATIC PATHWAYS OF DAMAGED NUCLEOTIDE EXCISION[1]

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ABSTRACT Py <> Py correndonucleases I and II from Micrococcus Luteus act exclusively on thymine-thymine, cytosine-cytosine, and thymine-cytosine cyclobutyl dimers in DNA, catalyzing incision 5' to the damage and generating 3'-hydroxyl and 5'-phosphoryl termini. Both enzymes initiate excision of pyrimidine dimers in vitro by correxonucleases and DNA polymerase I. The respective incised DNAs, however, differ in their ability to act as substrate for phage T4 polynucleotide ligase or bacterial alkaline phosphatase, suggesting that each endonuclease is specific for a conformationally unique site. The possibility that their respective action generates termini which represent different degrees of single strandedness is suggested by the unequal protection by Escherichia coli binding protein from the hydrolytic action of exonuclease VII.

INTRODUCTION

Perhaps the best understood pathways of DNA repair; - one involving a variety of nucleases is expressed as nucleotide excision either of nucleotides as first described by Carrier and Setlow (1) and Boyce and Howard-Flanders (2) or of bases as described by Lindahl (3). The initial recognition by site-specific nucleases which act on the primary chemical or photochemical damage is the rate-limiting step in the excision process. The other pathways, involving N-glycosylic bond hydrolysis by enzymes recognizing unique kinds of chemical changes, have recently received a great deal of attention and will be discussed at some length by Drs. E. Friedberg and T. Lindahl.

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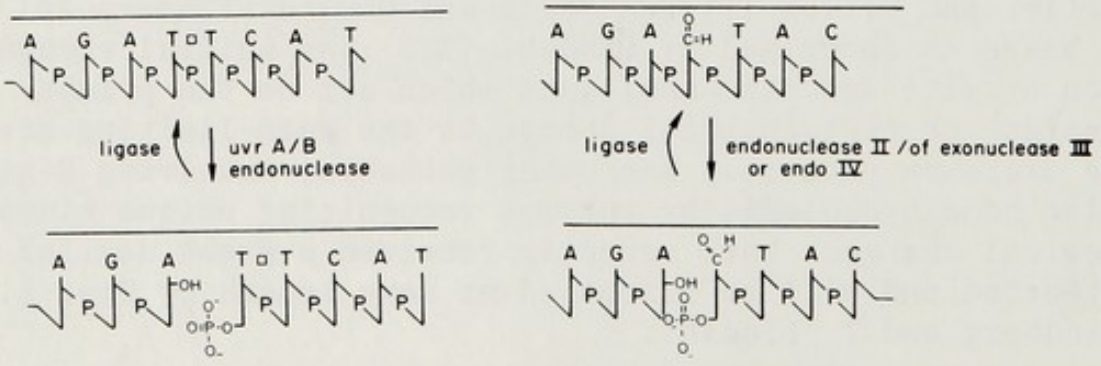
General Endonucleolytic Mechanisms - Nucleotide Excision. There have been a plethora of reports describing enzymes that act on damaged DNAs. There is a need for some distinction at this juncture between enzymes that act specifically at damaged sites and those that act as a secondary consequence of the distortion imposed upon the structure of DNA consequent to some prior modification. There have been reports in the literature, for example, that the Neurospora endonuclease (4) S_1 endonuclease (5) an endonuclease from Ustilago (6) and M. luteus (7) act on DNAs that have been heavily damaged with ultraviolet light as well as single stranded DNA. Such data suggest that there are endonucleases which may be distortion rather than damage base specific. The E. coli endonuclease V is a typical example of such an endonuclease whose role in repair is suggestive. The ultimate demonstration for the biological role of such enzymes depends upon the identification of the structural gene and its relationship to a specific gene product.

Nucleotide Excision - Damage Specific Endonucleases. There are three damage-specific endonucleases which act on pyrimidine dimer-containing DNA. The T_4 bacteriophage V^+ gene coded enzyme (7), the enzymes controlled by the uvrA, uvrB structural genes (8) and the enzymes isolated from Micrococcus luteus (9-11) behave in a similar manner. These enzymes are specific for the damaged strand (12) resulting in phosphodiester bond hydrolysis 5' to the damaged site generating 3'-hydroxyl and 5'-phosphoryl groups (12). Such sites are reversed with polynucleotide ligase by virtue of the juxtaposed 3' hydroxyl and 5' phosphoryl group (10,13). Although polynucleotide ligase has been used diagnostically in identifying these intermediate structures it is possible this "reversibility" may play an important role in vivo; this putative step being under the control of the uvrC gene (14).



(a) (b)

FIGURE 1. Damage Specific Endonucleases (a) pyr <>pyr correndonucleases (b) Apurinic/apyrimidinic endonucleases.

It is unclear why a controlling step is necessary at this point in repair. However, definitive binding data at such sites by competing enzymes, such as DNA polymerase I, and ligase is needed. From preliminary information concerning the competition of these two enzymes for nicked or incised sites, it can be suggested that ligase can limit polymerization or strand displacement reactions catalyzed by DNA polymerase I (15).

An endonucleolytic step is required to act specifically at sites generated by the DNA N-glycosylases(3-6). These apyrimidinic or apurinic sites act as substrates for the action of a number of ubiquitous endonucleases. In E. coli exonuclease III, its associated endonuclease activity (16) is able to act at such sites generating 5'-phosphoryl and 3'-hydroxyl termini, 5' to the deoxyribose moiety (17). It has been suggested by Verly (18) that the role of the exonuclease III activity in repair is for removal of a few juxtaposed nucleotides thereby limiting the action of ligase and thus abortive repair. Mutants of exo III/endonuclease II, (xth$^-$ mutants) possess a unique residual endonuclease activity free of associated exonuclease which acts specifically at apurinic sites. Endonuclease IV (19) has the capacity to act at such apurinic sites, presumably acting 5' to the damage to generate suitable sites for excision reactions.

Two enzymes have been isolated which appear to be specific for UV damage, one referred to as endonuclease III (20) and the other is the gene product of uvrA and uvrB (8). The latter enzymes are absent in excision-defective mutants whereas endonuclease III, requiring more heavily irradiated DNA is present in excision-defective mutants.

Endonuclease V recently reported by Gates and Linn (21) has a wide range of specificity; however its most interesting endonucleolytic property is its ability to act on DNAs containing uracil in place of thymine. The putative role of endonuclease V may be in metabolic repair under conditions in which uracil finds its way into DNA. The level of this particular enzyme in the recently isolated uracil N-glycosylase mutants (22) will be of interest.

Excision of Damaged Nucleotides. - DNA Polymerase-Associated Excision Enzymes. (Fig. 2) The bacterial polymerases with their associated exonucleolytic activities are involved in a number of important biological functions in addition to polymerization of nucleotides. Most notable is the 5' exonuclease activity specific for double stranded DNAs, which acts at nicks in an extremely efficient manner during concomitant polymerization. This nick translational activity of polymerase I functions by virtue of its nucleotide removal and reinsertion in a 5' direction. The ability

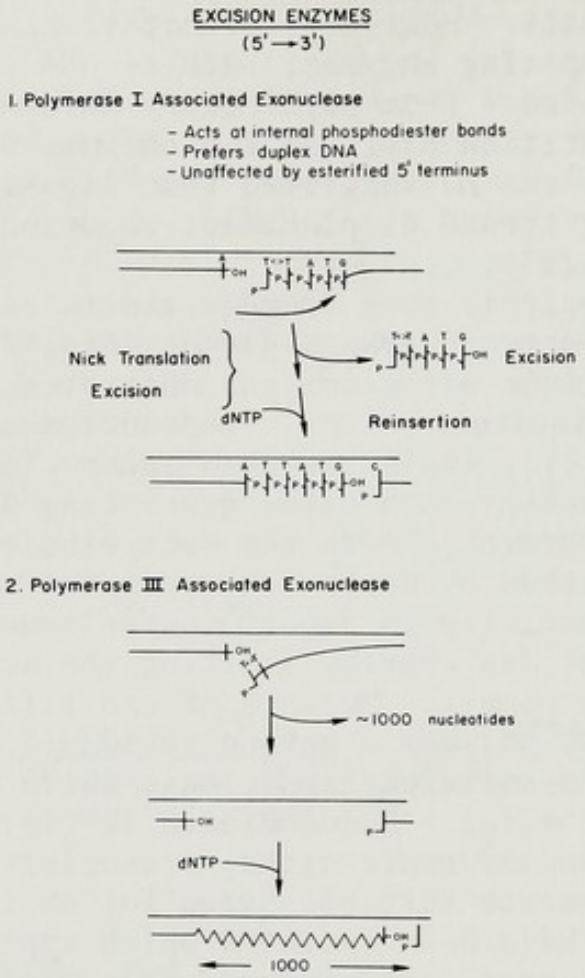


FIGURE 2. Polymerase-Associated Exonucleases in Excision

of this associated exonuclease to function at internal rather terminal phosphodiester bonds makes it suitable for the removal of 5'-terminal damaged nucleotides. This excision capability was first shown by Kelly et al. (23) to remove pyrimidine dimers from DNAs incised by nonspecific endonucleases. Similarly, damaged DNA treated initially by the correctional endonucleases from either M. luteus or from E. coli followed by polymerase I results in loss of pyrimidine dimer-containing fragments as an initial step during the excision reaction (15). This is a very efficient process because of the coordinated polymerization and excision by this multifunctional enzyme. In the absence of concomitant polymerization, excision is fairly sluggish. This was best demonstrated with the proteolytic digestion products of polymerase I (24) in which the smaller molecular weight fragment, although able to excise pyrimidine dimers, did so at diminished rates. Addition of the larger 77,000 molecular weight fragment which contains the polymerizing activity stimulated excision (25).

Polymerase III, according to Livingston and Richardson (26) possesses, in addition to its 3' editing function, a rather unique 5' exonuclease activity which requires that the 5' terminus exist in a noncomplementary state. Initiation of hydrolysis occurs at such a structure and then proceeds into duplex region in an uncontrolled way. From in vivo studies, the role of polymerase III in excision has not been convincingly implicated in repair.

Unassociated Exonucleases. A variety of unique exonucleases have been isolated from M. luteus, E. coli and from human placenta which act on single-stranded DNA but interestingly, initiate hydrolysis from either the 3' or 5' terminus, generating small 5'-phosphorylated oligonucleotides (27-29). These single stranded-specific exonucleases are also able to act on irradiated-incised duplex DNAs in an efficient manner. The placental correctional exonuclease can, in addition, digest at a nick in an undamaged duplex DNA in both a 3' and 5' direction generating a gap approximately 40 nucleotides in length (29). The bacterial enzymes, acting on incised-irradiated DNA generate a gap approximately 10 nucleotides in length (30), presumably because their activity is limited to single stranded-like regions of the incised DNAs.

Base - Nucleotide Incision Pathways. (Fig. 3) The nucleotide incision and base removal mechanisms coverage onto a common path. The initial removal of damage, catalyzed either

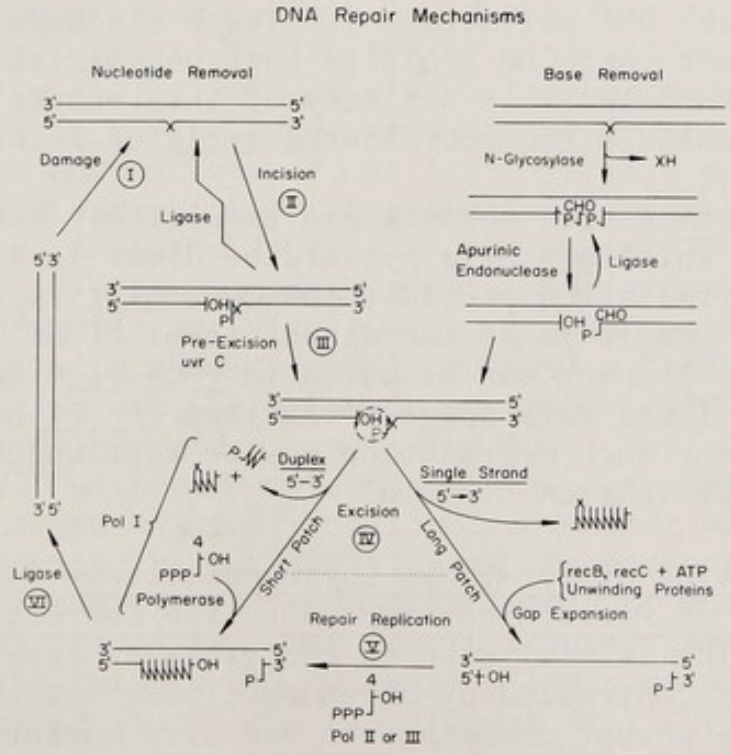


FIGURE 3. Nucleotide Excision Repair Mechanisms

by specific endonucleases acting at damaged sites or endonucleases acting at sites generated by DNA N-glycosylases lacking purines or pyrimidines are repairable by the efficient polymerase I excision-reinsertion pathway; finalized by strand restoration catalyzed by polynucleotide ligase. This most efficient pathway is mechanistically termed short patch repair (32). In polymerase I mutants the unassociated exonucleases are potentially capable of removing damaged oligonucleotides. By virtue of the rather short region available for either polymerase II or III in such mutants it can be suggested that a gap expansion step is necessary to allow for the processive binding and reinsertion by these two polymerases (32). This is the most likely pathway responsible for long patch repair. A discussion of the putative role of the rec BC exonucleases in this path has been made in a previous publication (33).

In either short patch or long patch repair accommodation must be made for preserving the integrity of the complementary strands during the transient stage in which vulnerable gaps exist following excision just prior to reinsertion. It has recently been shown that binding proteins inhibit the action of many nucleases which acdt at single stranded sites. It may be suggested that binding proteins can assume a protective role by inhibiting the action of nonspecific endonucleases at these sensitive sites (34,35).

In addition to the protective role of assumed by binding proteins, the E. coli protein (ECBP) participates facilitatively by increasing the rate and extent of nucleotide excision by the DNA polymerase I. These findings seem paradoxical in that the ECBP inhibits both polymerization and the 3'→ 5' exonucleolytic activity of this enzyme. yet significantly enhances the coordinated excision-reinsertion reaction (36).

RecA, uvrA double mutants are exquisitely sensitive to UV light, in which a single pyrimidine dimer is lethal (37). During postirradiation periods, the UV-sensitive single recA mutants result in an inordinant level of DNA degradation (reckless) which can be cured in recA recB double mutants (38). These data suggest that the rec BC nuclease responsible for such degradation may be kept under control by the protective recA protein.

Recognition of Pyr<> Pyr Correndonuclease - Structural Gene Control of Pyrimidine Dimer Specific Correctional Endonucleases. The initial step in the excision repair process in E. coli is controlled by an endonuclease activity which appears to be absent in both uvrA and uvrB mutants (8). The distal relationship of these two structural genes seems paradoxical in terms of the control of what appears to be a

TABLE 1
ORIGIN AND PHENOTYPIC PROPERTIES OF M. LUTEUS STRAINS

Strain	Isolation	Percent Survival After UV (50 J/m^2)	Resistance to mitomycin (0.05 g/ml)
Wild Type	ATCC #4698	100	+
DB-7	wild type cells NTG mutagenized	0.01	-
DB-200	DB-7 transformed with wild type cells	0.10	-
DB-400	DB-200 transformed with wild type cells	100	+

a single enzyme. A similar situation exists in M. luteus although unmapped genetically. It is apparent that similar enzymatic activity is controlled by at least two genes in this organism (11). It seemed advantageous to investigate this problem in M. luteus because of the greater concentration of such endonucleolytic activity in this organism. An analysis of a series of mutants and transformants of this organism indicates (Table 1) that the mutants which are obtained through nitrosoguanidine mutagenesis are UV sensitive and lack enzymatic activity (DB7). Transformation of such mutant cells with wild-type DNA produced a transformant which is UV sensitive but which has demonstrable enzymatic activity when measured in crude extracts (DB200). Further transformation of DB200 with wild type DNA yields a revertant which is UV resistant and has fully restored enzymatic activity (DB400). During the purification of the enzymes on phosphocellulose chromatography wild type levels of enzymes assayed with heavily irradiated DNAs were partially resolved (peaks I and II). The mutant, DB7 possessed little or no activity; DB200 the first transformant, although still UV sensitive, possesses one peak restored to wild type levels. DB400, the revertant possess wild type levels of both peaks of activity.

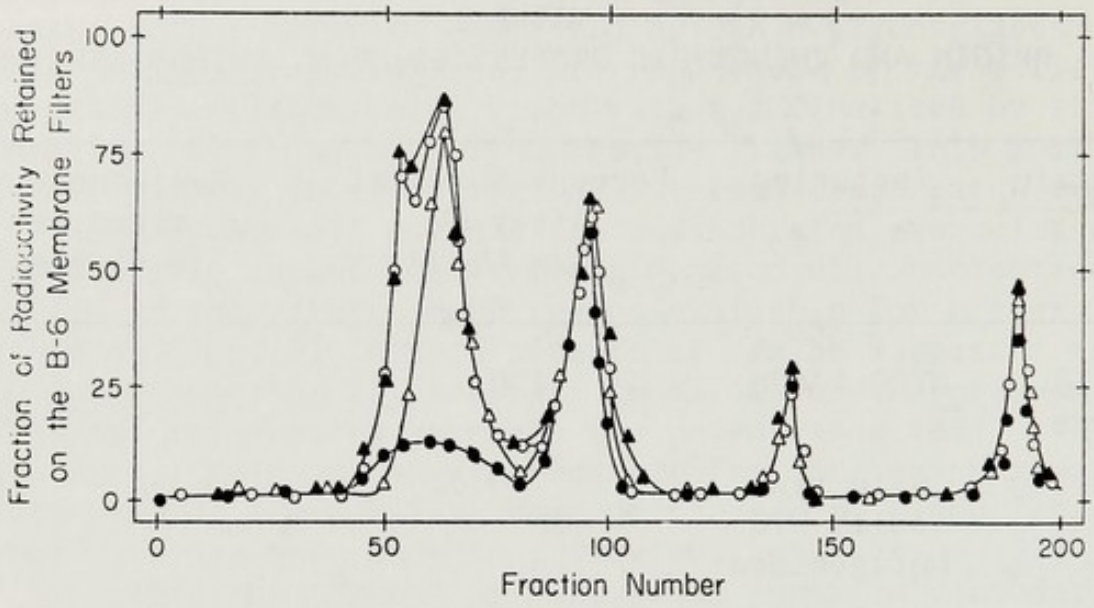


FIGURE 4. Phosphocellulose Chromatography of M. Luteus Strains

The third, fourth and fifth peaks were used for reference for further identification of enzyme activities which recognize heavily irradiated DNA. Their persistence in all of the mutants and transformants tested is diagnostic for their lack of involvement in ultraviolet damage repair process in these organisms. (Fig. 4)

An analysis of the same enzymatic activities at doses which include no more than three to four pyrimidine dimers per genome is seen in which the presence of peaks III, IV and V are not evident until DNAs have been more heavily irradiated. The important feature of this finding is that the first two peaks of activity are both present when either heavily or lightly irradiated DNAs are used as substrate. (Fig. 5)

The assay procedures employed for these experiments measure the conversion of irradiated ØX174 RFI to RFII species (39). This has the further virtue in permitting measurements of the binding properties of these enzymes to damaged DNA regions. Through an appropriate ionic environment the supercoiled DNA is not retained on nitrocellulose filters whereas protein is. It is useful to measure the enzyme-substrate complex in a manner analogous to that of repressor binding (40). An analysis of the phosphocellulose chromatograms for the binding properties of these enzymes indicated that peaks I and II exhibit differential binding to irradiated RFI DNA. By taking advantage of this binding difference resolution of the enzymes is achieved by affinity (DNA-cellulose) chromatography. Final purification of these

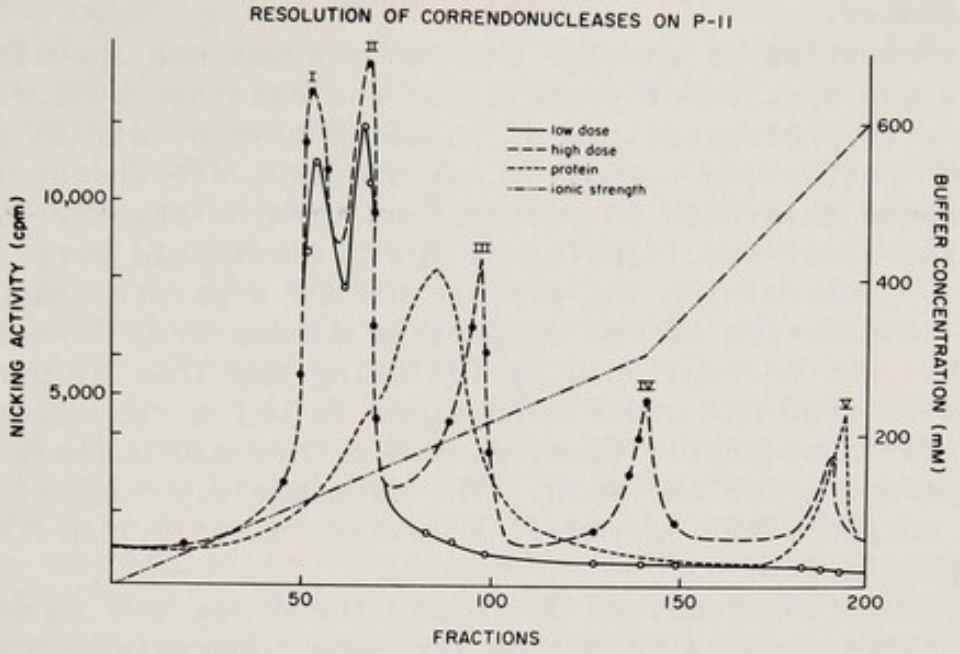


FIGURE 5. Phosphocellulose Chromatography of M. Luteus Endonucleases

two peaks on isoelectric focusing revealed that peak I differs in its isoelectric point of 8.7 from peak II which is more acidic with an isoelectric point of 4.7.

A comparison of the substrate saturation curves clearly showed that peaks I and II reach saturation with no more than 2 to 4 pyrimidine dimers per genome. Peak III, IV and V however, required DNA substrates with higher levels of modification before saturation was obtained. These differences distinguish the damage-specificity of peaks I and II from the damage-dependency of the other three peaks of activity. Peak III has a wide range of specificity including λ irradiated DNA, alkylated-depurinated DNA, DNA treated with OsO_4 (which forms a thymine glycol derivative analogous to a gamma irradiation product (41) and undamaged denatured DNA. This endonuclease probably recognizes the common secondary effects arising from the diverse form of damaging agents.

That two enzymes are specific for UV-irradiated DNA demanded a resolution of their substrate specificities. The ability of either peaks I or II to act specifically on pyrimidine-pyrimidine dimers was examined. The action of photolyase on irradiated DNAs (photolyase monomerizes T<>T, C<>T and C<>C pyrimidine dimers), is able to reverse the action of the two enzymes implicating pyrimidine dimers as the substrate on the DNA for these enzymes. Incubation of irradiated DNA with this enzyme in the absence of light

however, does not affect the substrate capabilities of such DNA molecules.

The question of whether the two enzymes are specific for a unique species of pyrimidines was examined by irradiating DNA with photosensitizers, in which thymine dimers are the major photoproduct and C<>T and C<>C are absent (42). It was found that both enzymes act on thymine-thymine dimers. Pyrimidine dimer specificity was further examined more closely by determining the effects of the separate endonucleases on excision of the respective dimers from irradiated DNA. This was accomplished by following the loss of pyrimidine dimers from DNA under conditions in which the endonuclease was subsequently treated with either exonuclease VII or polymerase I for excision. The data clearly suggest that neither enzyme I nor enzyme II show any unique pyrimidine dimer specificity.

The question remained as to why there are two separate endonucleases seeming to act on the same substrate. Furthermore, genetic analyses indicated that both proteins are required to confer UV resistance on cells.

The possibility that these two enzymes may be acting in different regions of the DNA molecule was pursued. The irradiated DNAs were incised by each endonuclease and the structure of the sites generated in each case was studied. Three different experimental approaches were utilized, all of which are diagnostic for the appearance of single stranded-like regions at the site of incision. The first procedure utilized bacterial alkaline phosphatase (BAP) which is heat stable and rather large protein. Weiss et al (43) formerly showed that BAP cannot act directly at a nick but requires prior denaturation of DNA in order for the phosphomonoester group to become available to the action of BAP. The heat stability of the BAP permitted the study of the availability of such termini in an environment of changing temperature. From such experiments it is apparent that the phosphomonoester sites generated by correndonuclease I are significantly more accessible to the action of BAP than is that site generated by correndonuclease II. These data are consistent with two unique sites being generated.

It was assumed that a distinction between these termini could be made by examining the susceptibility of these nicks to polynucleotide ligase. Ligase can catalyze the formation of a phosphodiester bond even though the 3' juxtaposed nucleotide is noncomplementary (44). The rates of ligation with DNAs incised with correndonuclease I were six times slower than those obtained with correndonuclease II or pancreatic DNase I. These findings identify distinctions between the two products of incision and that DNA incised with

enzyme I is converted less rapidly to RFI in comparison to that generated by correndonuclease II. These data are in keeping with the suggestion that the site generated by correndonuclease I is more single stranded-like than that generated by the second enzyme.

An additional approach to studying these structural differences, employed the co-operative binding properties of the E. coli binding protein to single stranded DNA. In order to assess the effects of binding protein use was made of an observation in which single-stranded DNA associated with binding protein is resistant to the action of exonucleases VII (10). In these experiments the incised irradiated DNA was preincubated with stoichemetric amounts of binding protein and subjected to hydrolysis by exonuclease VII. The data show that the site generated by enzyme I, when 25% hydrolyzed by exonuclease VII, causes complete shutoff of further hydrolysis. The site generated by correndonuclease II is quantitatively hydrolyzed although at a reduced initial rate. These data support the suggestion that unique sites are generated by the resolved correndonucleases.

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ENZYMOLOGY OF NUCLEOTIDE EXCISION REPAIR[1]

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Elimination of damaged nucleotides by excision repair involves two main enzymatic activities: a damage specific endonuclease which recognizes the damaged site and introduces a nick in the vicinity of the damage, and an exonuclease which releases the damaged nucleotide/s introducing a second nick at the other side of the damage.

ENDONUCLEOLYTIC ACTIVITIES

A number of damage specific endonucleases have been isolated from different sources and some of them are extensively purified and characterized. No satisfying classification, nevertheless, exists since only in the case of dimer-specific endonucleases does the mechanism of damage recognition seem to be understood. For this reason, in addition to dimer-specific enzymes (group I), endonucleases acting on DNA damaged by different agents will be divided into enzymes which are damage specific only (group II) and those which act also on untreated DNA (group III).

I. Dimer-Specific Endonucleases. Such enzymes have been purified from M. luteus (1-4) and phage T4 infected cells (6-8). These small enzymes (M.W. 11.000-18.000) are active in the presence of EDTA and do not require any cofactor or sulfhydryl agent for its activity. They do not attack DNA containing damage other than cyclobutyl pyrimidine dimers and the number of nicks they introduce corresponds to the number of dimers in the molecule. Both enzymes incise UV-irradiated single- and double-stranded DNA and nick the dimer-containing strand in heteroduplex DNA containing a UV- and unirradiated strand (2,8,9). These data imply that the chemical structure of the dimer rather than the local distortion of the helix is recognized by the dimer-specific endonucleases.

A different type of endonuclease, which apparently act on dimers, has been found in E. coli cells (10,11). This uvrA-, uvrB and uvrC-dependent enzyme requires ATP and Mg^{2+} for its

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activity. The gene products have been partially purified using a complementation assay and the endonucleolytic activity was reconstructed *in vitro* (12). The *uvrA*-product has a molecular weight of 100.000 and shows properties of a DNA binding protein (13). The copurifying *uvrB*- and *uvrC*-products have a molecular weight of 70.000. This number may reflect the size of the *uvrB*-product or that of a complex of the copurifying products. Neither of the gene products is an endonuclease and all three are needed for the endonucleolytic activity of the complex (12).

From *E. coli* two additional endonucleases have been isolated one of which has also been attributed to the *uvrA* and *uvrB* genes (14-16). They are small (M.W. 12.000 and 20.000) ATP-independent enzymes active in the presence of EDTA. The smaller enzyme binds to DNA treated with 900 J/m^2 and photoreactivation diminishes this binding. In conjunction with exonuclease VII it preferentially releases dimers from UV-irradiated DNA.

The existence of two different types of UV-dependent endonucleases can be explained taking in account data obtained with permeabilized cells (10,14). The high molecular weight ATP-dependent activity reconstructed in vitro (12) corresponds well with the uvr-gene-dependent activity which incises UV-irradiated DNA in permeable *E. coli* cells incubated in the presence of ATP (10,17). The relatively low ATP-independent activity observed in all uvr^+ and uvr^- strains tested (10,17) may reflect the small molecular weight ATP-independent activity (14, 15).

That several activities recognizing dimers may be present in one and the same cell appears also from studies with *M. luteus* which reveal two distinct dimer-specific activities (18). Each enzyme recognizes dimers *in vitro* but both are needed for successful repair of UV-damages *in vivo* (19).

An endonuclease which acts on DNA irradiated with low UV-doses has been recently isolated from calf thymus (20). This Mg^{2+} dependent enzyme which copurifies with a high molecular weight unspecific endonuclease is highly labile after being isolated from the tissue. It seems to recognize dimers since the number of nicks it makes is comparable to the number of dimers in the DNA. This is the first mammalian UV-endonuclease which shows such properties. All the previously isolated mammalian endonucleases recognize lesions other than dimers (21-24; see group II endonucleases).

UV-dependent activities have also been partially purified from *B. subtilis* (25), *Dictyostelium discoideum* (26) and *Schizophyllum commune* cells (27) but not enough data exist to decide if they are dimer-specific.

In summary, two types of dimer-specific endonucleases are found: i) Mg^{2+} independent low molecular weight enzymes and

ii) Mg^{2+} dependent enzymes which are high molecular weight proteins or protein complexes. The yet nonisolated freeze-sensitive human activity possibly belongs to the second type of dimer-specific endonucleases.

II. Endonucleases that Act on DNA Treated with a Variety of Agents. Such enzymes isolated from a variety of sources are mostly called x-ray- or UV-endonucleases depending which of the agent was in the center of interest of the investigator.

The E. coli x-ray-endonuclease nicks DNA treated with x-rays, OsO_4, low pH or high doses of UV-light (28,29). In DNA treated with x-rays in the presence of ion scavengers which increase relative amount of base damage versus strand breaks, this enzyme recognizes alkali labile (apurinic sites), slow converting alkali labile and alkali stabile lesions. The enzyme does not require Mg^{2+} and has a pH optimum close to neutral. Two other independently isolated small molecular weight enzymes (30,31) one of which is known as E.coli endonuclease III (31), show similar substrate specificities. The three enzymes have much in common and possibly represent one and the same enzyme.

Similar activities have been purified from rat liver (21), calf thymus (22) and human lymphoblasts (23,24). These mammalian endonucleases have a molecular weight close to 30.000 and do not require Mg^{2+}. They act on x-irradiated DNA and DNA treated with high doses of UV-light. In the latter they introduce only a limited number of nicks,much lower than the numbers of dimers present. They seem to recognize base damages in x-irradiated DNA and minor photoproduct/s in UV-irradiated DNA. Their activity at alkali labile sites which apparently are apurinic/apyrimidinic sites may belong to a copurifying endonuclease since additional purification of the human enzyme separated an apurinic site specific enzyme from the main activities of this enzyme (32).

That enzymes acting on x-irradiated DNA need not be necessarily active at apurinic sites follows also from the properties of a M. luteus γ-endonuclease (33,34). Unlike its main γ-ray-dependent activity, which requires Mg^{2+} and is salt sensitive, its activity on depurinated DNA is preserved in the presence of EDTA and 100 mM KCl. Another γ-endonuclease from the same source (35), is active on γ-irradiated, OsO_4 treated and depurinated DNA. It is unclear how far the differences described reflect incomplete purification or the existence of several distinct enzymes. The latter correlates with M. luteus chromatography data showing three distinct peaks of activity all of which act on DNA treated with x-rays, monoalkylation agents and high doses of UV-light (4).

More data are needed to decide what do group II damage specific endonucleases recognize as a damage. They may recog-

nize a certain common base defect or a secondary distortion of the helix induced by a variety of agents. Endonucleases which recognize 5,6,-dihydroxy-dihydrothymine induced by such different agents like OsO_4, x-irradiation and high doses of UV-light (33), may belong to the first kind of enzymes. Endonucleases which also act at apurinic sites may represent enzymes of the second kind.

III. Endonucleases that Act on Untreated and Damaged Duplex DNA. Such enzymes have been isolated from Neurospora crassa (37), Aspergillus oryzae (38) and E. coli cells (39). These single-strand specific enzymes are limited active on untreated duplex DNA. Irradiation with high doses of UV-light turns double-stranded DNA into a better substrate for these enzymes. The recently isolated E. coli endonuclease V (39), which is a small molecular weight Mg^{2+} dependent enzyme, nicks also DNA treated with low pH or OsO_4. This E. coli enzyme is especially peculiar in incising phage PBS2 DNA which contains uracil. The production of nicks at uracil containing sites is a unique property of this enzyme and has not been described for any other endonuclease.

These single-strand specific enzymes seems to recognize a locally denatured region created in the double-stranded DNA by the variety of agents listed. The recognition of uracil by the E. coli enzyme is obviously related to some other recognition principle.

EXONUCLEOLYTIC ACTIVITIES

Most of the damage-specific endonucleases, if not all, introduce 3'OH and 5'P-terminated nicks at the 5'side of the damage. This suggests that a 5'-3' exonucleolytic activity is needed to remove the damaged region from the DNA.

Some cell types possess several 5'-3' exonucleolytic enzymes. E. coli harbors four such activities. Two are associated to DNA polymerase I and DNA polymerase III (40). Exonuclease VII also preferentially releases dimers from UV-irradiated DNA (41). The ATP-dependent exonuclease V also excises dimers (42). Unlike the other E. coli exonucleases which start at nicks, this enzyme releases dimers only after a gap has been created in the vicinity of the dimer. It is unclear how far this enzume plays any role in dimer excision in vivo.

Two 5'-3' exonucleolytic activities which remove dimer containing oligonucleotides are purified from phage T4 infected cells (43), and two 5'-3' directional exonucleases are purified from M. luteus cells (1,44). One of them is an unassociated exonuclease, the other is associated to the DNA polymerase. A dimer excising enzyme has been isolated from human placenta (45). Mammalian cells seem to possess several 5'3'exonu-

cleolytic-activities. Since three different dimer excision activities were recently isolated from a human cell line (46).

A 3'-5'exonucleolytic activity must be involved in damage removal from x-irradiated DNA. Along with base damages x-rays induce strand breaks most of which do not have 3'OH termini. These "unclean" ends have to be "cleaned" by an exonuclease since only 3'OH ends are primer sites for DNA polymerases. An exonucleolytic priming activity which prepares x-irradiated DNA for a DNA polymerase has been recently isolated from B. subtilis cells. (47). Exonuclease III does the same in vitro (48).

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A DNA-BINDING ACTIVITY ASSOCIATED WITH THE *uvrA*$^+$ PROTEIN FROM *Escherichia coli*

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ABSTRACT An *in vitro* complementation assay has been used for the partial purification of the *uvrA* protein from *E. coli* (1). The *uvrA* protein is recovered in a high molecular weight form (MW ~ 100,000) and has no detectable endonuclease activity, but is associated with a DNA-binding activity with preferential affinity for UV-irradiated DNA. This binding activity is absent from a *uvrA* mutant strain and therefore is possibly an inherent property of the *uvrA* protein itself.

INTRODUCTION

The gene functions coded for by *uvrA*$^+$, *uvrB*$^+$ and *uvrC*$^+$ in *E. coli* are jointly required for the removal of several types of DNA damage induced by ultraviolet light (UV), bifunctional alkylating and DNA crosslinking agents (for review see ref. 2). Mutants defective in these genes were first described by van de Putte *et al.* (3) and Howard-Flanders *et al.* (4) and characterized as being unable to excise UV-induced pyrimidine dimers from the DNA (4,5). Later studies of DNA strand breakage in UV-irradiated cells have indicated that the *uvr*-genes control an endonuclease activity which recognizes pyrimidine dimers in the DNA and induces breaks in the vicinity of the lesions in the DNA strands (5,6). However, the specific role of each one of these gene products in this process is still unknown, mainly due to difficulties encountered in purifying the *uvr*$^+$-coded endonuclease. We have recently introduced a new approach for the isolation of the *uvr* gene products which aims at the separate purification of each one of these by means of an *in vitro* complementation assay (7,1). This approach avoids the difficulty arising when a multi-subunit enzyme becomes resolved during purification, since each one of the *uvr* gene products can be detected separately by means of the complementation system. By using this approach, the *uvrA*$^+$, *uvrB*$^+$ and *uvrC*$^+$ gene products have been partially purified and a UV-endonuclease activity has been reconstituted from the partially purified components (1). This activity induces approximately one break for each dimer present in the DNA, and is completely ATP-dependent as could be suspected from previous

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studies of permeable cells (8,9) and crude extracts (7). ATP-requirement has not been described for other repair endonucleases and seems characteristic for the uvr$^+$-dependent enzyme from E. coli.

In this communication we describe one property of the uvrA$^+$ protein which may be suggestive of the function of the uvrA$^+$ protein in the endonuclease reaction.

RESULTS AND DISCUSSION

Results from a gel filtration experiment with partially purified uvrA$^+$ protein is shown in Fig. 1. The uvrA$^+$ protein

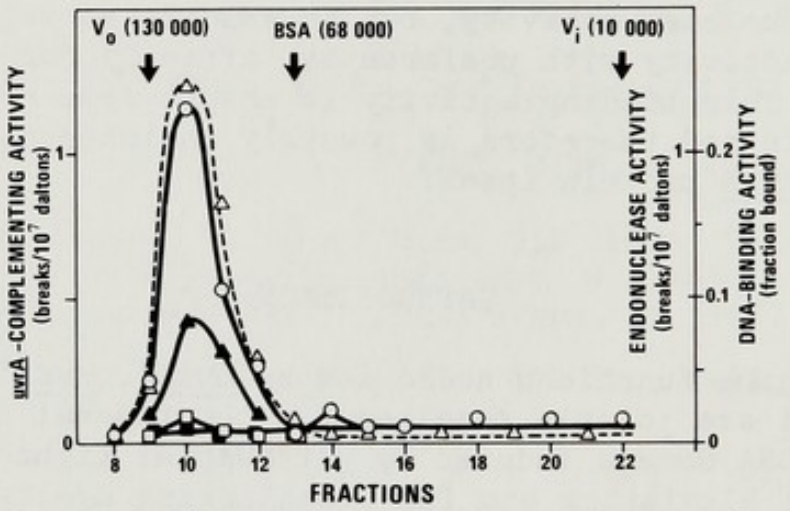

FIGURE 1. Gel filtration of the uvrA protein.

Partially purified uvrA$^+$ protein prepared as described (1) was applied to a polyacrylamide-agarose gel column (20x1 cm, Ultrogel ACA 44) equilibrated with 300 mM KCl in buffer A (50 mM MOPS, pH = 7.5, 10 mM mercaptoethanol, 1 mM EDTA, 25% glycerol). Eluting fractions (0.7 ml total, 10 µl of each) were tested for uvrA-complementing activity (ref. 1) (○), endonuclease (squares) and DNA binding (triangles) activity on native (open) and UV-irradiated (180 J/m^2 of 254 nM light, closed symbols) DNA. Endonuclease activity was measured by the filtration assay of Center et al. (10) as modified (11,1) using reaction conditions as described below except that samples were incubated for 15 min. at 37°C. In the binding assays 0.05 µg DNA, 100 µl buffer (100 mM KCl, 50 mM MOPS, pH = 7.5, 20 mM $MgSO_4$, 1 mM ATP, 1 mM EDTA, 1 mM dithiothreitol) and protein fraction were mixed, incubated 5 min. at 30°C, and 15 min. at ice temperature before addition of 2 ml icecold 200 mM KCl, 50 mM MOPS, pH = 7.5, 10 mM $MgSO_4$ and slow filtration through Millipore (HAWP) filters. The fraction of radioactivity retained on the filters after washing was plotted as DNA binding activity. Endonuclease assay without Mg^{2+} and ATP in the reaction mixtures gave the same results as shown for assays with these components added.

has an apparent molecular weight of 100,000 and is free of detectable endonuclease activity on non-irradiated as well as UV-irradiated DNA. However, the uvrA$^+$ protein comigrates with a DNA-binding activity with preferential affinity for UV-irradiated DNA. This binding activity is stimulated by a short incubation at 30-37°C and requires the presence of Mg^{2+} both during this incubation and during subsequent dilution and filtration to measure the DNA protein complex. Also ATP stimulates the binding activity, but not specifically as GTP has a similar effect. This contrasts the ATP-dependence of the uvrA$^+$, uvrB$^+$, uvrC$^+$-coded endonuclease activity which seems quite specific since neither GTP nor ADP or AMP can substitute for ATP in the endonuclease reaction (8,12).

To ensure that the binding activity was associated with the uvrA$^+$ protein and not a contamination in the partially purified preparation, purification of the binding activity was attempted from a uvrA mutant strain in parallel with wild type cells. As shown in Fig. 2 both the complementing and the binding activity is absent from the uvrA mutant strain. It seems likely therefore that this binding is caused by the uvrA$^+$ protein itself, or alternatively by a factor complexed with the uvrA$^+$ protein.

The UV-DNA binding property of the uvrA$^+$ protein may be important for the function of the uvrA$^+$ protein in the UV-endonuclease reaction. The uvrA$^+$ protein does not seem to bind to pyrimidine dimers per se as the binding is significant also to non-irradiated DNA. It is possible that the binding protein has affinity for unpaired base regions which is known to be present in superhelical covalently closed DNA and to a greater extent in DNA exposed to UV. It might be speculated that the

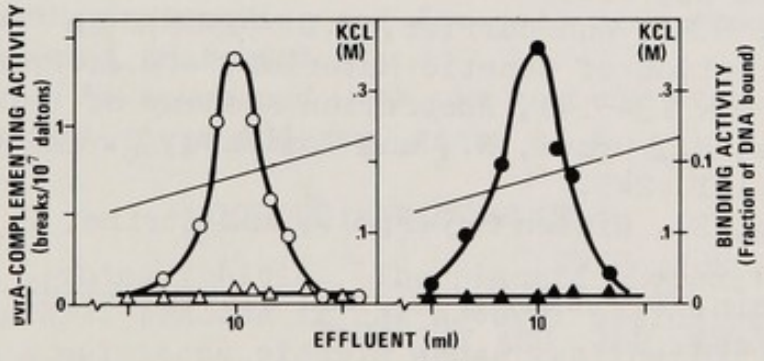

FIGURE 2. Phosphocellulose chromatography of protein extract from strains AB1157 uvr$^+$ and AB1886 uvrA.

Protein extracts from $2x10^{11}$ cells (1) were applied to phosphocellulose columns (1x7 cm, Whatman P11) equilibrated with 100 mM KCl in buffer A. The columns were washed, eluted with 100-300 mM KCl in buffer A and fractions assayed for uvrA-complementing (1) (left) and DNA binding activity on UV-irradiated DNA (right) as described in legend to Fig. 1. Symbols: circles, AB1157, triangles, AB1886.

function of the uvrA$^+$ protein is to adjust the conformation of the DNA at the dimer site to make it accessible for the action of the other uvr gene products. It is emphasized, however, that there is no direct experimental support of these speculations and that a closer investigation of the DNA target for and chemical nature of the binding is necessary before the significance of the binding activity can be evaluated.

A DNA binding activity with preferential affinity for UV-irradiated DNA has previously been described for a UV-endonuclease which appeared to be absent from uvrA and uvrB mutant cells (11). This enzyme is of low molecular weight (~12,000) and seems different from the binding activity described here which is of high molecular weight and without associated endonuclease activity. The relationship between the E. coli enzyme previously described and the one reported here is unclear and further work is required to clarify these apparent discrepancies.

ACKNOWLEDGEMENTS

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CLONING OF *E. COLI* DNA REPAIR GENES[1]

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ABSTRACT Plasmids carrying the *uvrA*, *uvrB* and *uvrC* genes of *E. coli* have been obtained. The plasmid pBR322 was used for the cloning of the *uvrA* and *uvrB* genes using standard methods for producing recombinant molecules. These plasmids replicate under relaxed control to form many copies per cell. The plasmid carrying *uvrC* is an F' that is present in cells at a frequency of about 1 per chromosome.

INTRODUCTION

Recent advances in the methodology of recombinant DNA research make it feasible to clone many interesting genes. Cloning of DNA repair genes should help in the assignment of gene products to their structural genes. It is also anticipated that the increased yields of proteins coded for by cloned genes will speed up the elucidation of their functions. Cloned genes are also excellent sources of DNA for sequence determinations and for cell-free studies of transcription and translation.

This paper is concerned with the current status of cloning the *uvrA*, *uvrB* and *uvrC* genes of *E. coli*.

METHODS AND RESULTS

Choice of a Cloning Vehicle. The plasmid chosen for these studies is pBR322 because it has several useful properties (1). It is a multicopy plasmid whose replication is under relaxed control meaning that there are normally more than 20 plasmid molecules for each bacterial chromosome. The plasmid DNA can be further amplified by growth of the cells in chloramphenicol. DNA inserted into the plasmid will be

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replicated under control of the plasmid and will also be amplified.

pBR322 was derived so that it contains single restriction sites for 5 different restriction endonucleases in regions of the plasmid that are not essential for its replication. All of these enzymes (EcoRI, HindIII, BamHI, SalI and PstI) can be used for the insertion of DNA fragments because they generate complementary ends of 4 nucleotides. The probability is thus very high that one or more of these enzymes can be used to clone a specific gene because it is unlikely that a gene will contain restriction sites for all 5 enzymes. In addition, combinations of 2 separate restriction enzymes can be used to clone a specific gene if neither of them cleaves the gene or its promoter. This should be particularly advantageous in reducing the amount of "uninteresting" DNA that flanks the specific gene being studied. Reducing this extraneous DNA may be essential for assigning gene products to their structural genes as it should greatly eliminate the possible confusion introduced by products of nearby unrelated genes .

Selection of recombinant DNA molecules after transformation is facilitated because pBR322 carries resistance to the antibiotics tetracycline (Tc) and ampicillin (Ap). Having 2 different antibiotic resistance characters is important because all of the restriction enzymes except EcoRI inactivate one of the markers when a recombinant DNA is formed. In these cases, the second intact antibiotic resistance character remains to be used for selection even though one is lost as a consequence of the cloning procedure.

Screening of the Clarke-Carbon Collection of ColE1 Hybrid Plasmids. Clarke and Carbon (2) have prepared a series of about 2000 plasmids in which random fragments of *E. coli* chromosomal DNA with an average size of about 10 Mdaltons were inserted into the ColE1 plasmid. In principle, most *E. coli* genes should be carried by one or more plasmids in this collection, but the problem is that only a small proportion of the plasmids have been characterized with regard to identifying the particular *E. coli* segment that is present in individual plasmids.

Since the host strain for these plasmids is F^+ and sensitive to streptomycin (Sm^s), the ColE1 recombinant plasmids can be transferred to F^- *uvr*$^-$ recipients that are resistant to streptomycin (Sm^r). We did matings of all 2000 strains with 3 recipients that were mutant at one of the 3 loci, *uvrA*, *uvrB*, or *uvrC* and looked for transfer of the UV-resistant phenotype. Although this method is tedious and has a substantial background noise level, we were able to obtain a ColE1 plasmid pLC 25-23 that carries the *uvrB-bio* segment of the *E. coli* chromosome as discussed below.

In addition, several plasmids in this collection have been reported to carry the *fiaD* gene which is adjacent to *uvrC* on the *E. coli* map. In our experiments reported in the *uvrC* section, complementation between these plasmids and *uvrC34* was not observed.

A Plasmid Carrying the uvrA Gene: pDR2000. Standard recombinant DNA methods (3) were used to obtain a plasmid carrying the *uvrA* gene of *E. coli*. Total DNA was prepared from *E. coli* K12 strain CGSC4260 that has an F' with the *uvrA* gene. This DNA was mixed with pBR322 DNA, cleaved with the *EcoRI* restriction endonuclease, and then was ligated with T4 polynucleotide ligase.

The ligated DNA was then used to transform (4) strain CSR603 (*uvrA6 recA1 phr1*). Colonies that were resistant to UV and to tetracycline were examined for the presence of a recombinant plasmid. A recombinant plasmid designated pDR2000 was obtained. This plasmid DNA was purified and used to transform CSR603. After transformation with this purifed DNA, cells simultaneously acquired resistance to both UV and tetracycline confirming the formation of a recombinant plasmid carrying the wild-type *uvrA* gene. The UV survival of strain CSR603 with and without this plasmid is shown in Figure 1a.

pDR2000 has a size of about 8.5 Mdaltons. Cleavage of pDR2000 with *EcoRI* produces 3 fragments of sizes 1.6, 2.6 and 4.3 Mdaltons. The 2.6 Mdalton fragment is the original plasmid pBR322 while the other 2 fragments are of bacterial origin. It is probable that the 2 chromosomal fragments came from separate parts of the bacterial chromosome and were fortuitously linked during the *in vitro* recombination. The plasmid has also been cleaved with *PstI* and mixtures of *PstI* and *EcoRI*. The results of these digestions are summarized in the restriction map in Figure 2.

In an attempt to reduce the size of the pDR2000 plasmid, the 1.5 Mdalton fragment generated by double digestion of pDR2000 with *EcoRI* and *PstI* was cloned, but after transformation with this smaller plasmid, a *uvrA* strain remained UV-sensitive. The most likely interpretation of this result is that the *uvrA* gene is located entirely on the 4.3 Mdalton *EcoRI* fragment.

A Plasmid Carrying the uvrB Gene: pDR1472. During the screening of the Clarke and Carbon plasmid collection, we found that transfer of pLC25-23 to a *uvrB5* strain cause it to become resistant to UV. pLC25-23 DNA was used to transform a *recA* strain 152Δ which has a deletion covering the *uvrB* gene and the *bio* operon (5). The transformed strain was resistant to UV and grew in the absence of biotin demonstrating that the chromosomal fragment in pLC25-23 contained *uvrB* and the *bio* operon. This finding was of interest not only because pLC25-23 carries the *uvrB* gene but because an

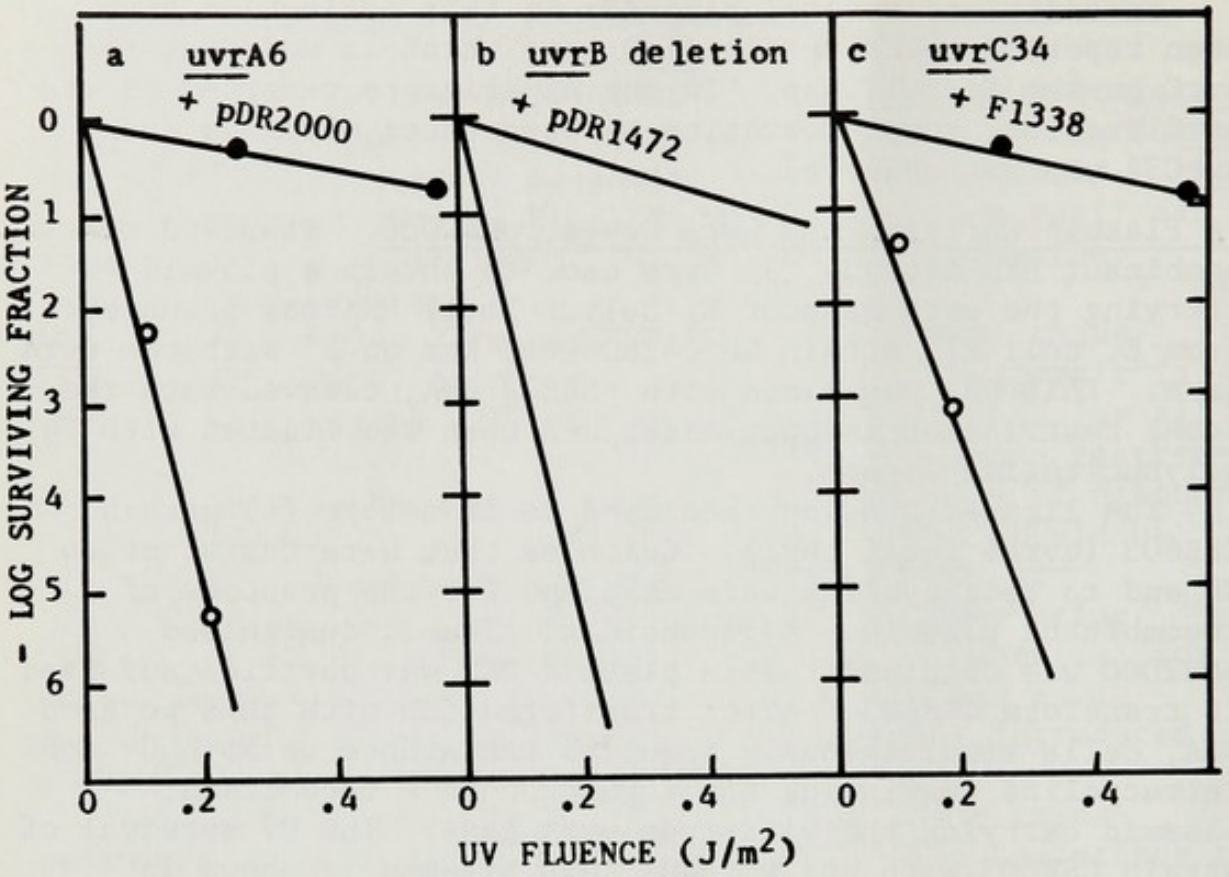

FIGURE 1. Effect of plasmids carrying uvr genes on the survival of UV-irradiated E. coli strains with uvr mutations. All strains also have a recA mutation in addition to the uvr defect indicated in the figure. The curves in panel b were extrapolated from results obtained at higher fluences.

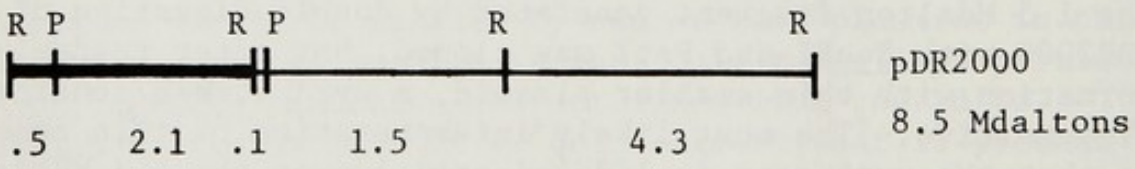

FIGURE 2. Restriction map of pDR2000. The heavy line represents the original pBR322 and the light line corresponds to the cloned fragments carrying the uvrA gene. The circular map has been opened at the site of insertion for convenience. The letters R and P indicate the sites of cleavage by the EcoRI and PstI restriction nucleases while the numbers give the distance in Mdaltons between restriction sites.

earlier study suggested that it was not possible to clone bio genes using ColE] as a vector (6).

The size of pLC25-23 is about |6 Mdaltons of which ColE| comprises 4.2 Mdaltons. In order to reduce the size of the plasmid and also to transfer the uvrB gene to the more convenient vector pBR322, DNA's from pBR322 and pLC25-23 were mixed, treated with PstI endonuclease and ligated with T4 ligase. The DNA was used to transform strain ↓52Δ to resistance to UV and tetracycline. The transformant was sensitive to ampicillin as expected because cloning with PstI inactivates the Ap^r segment of pBR322. Plasmid DNA was isolated from this strain and was used for further transformation of strain 152Δ. Resistance to both UV and tetracycline occurred at a high frequency confirming the presence of the uvrB gene on this recombinant plasmid designated pDR1472. The effect of pDR1472 on the UV sensitivity of strain 152Δ is shown in Figure 1b.

The plasmid pDR1472 has a size of about 9.4 Mdaltons. The preliminary restriction mapping indicates that there are 2 PstI fragments of about 3.4 Mdaltons in addition to the original pBR322. This plasmid thus contains only about half of the chromosomal DNA present on the original plasmid pLC25-23.

A Plasmid Carrying uvrC: F1338. At present we are attempting to construct a derivative of pBR322 that carries the uvrC gene. Since we have not yet identified a multicopy plasmid with the uvrCgene, we are using purified F1338 plasmid DNA as the source of the uvrC gene. The plasmid described by Silverman et al (7) decreases the UV-sensitivity when present in a uvrC34 recA1 strain constructed in our laboratory (Figure 1c). F1338 is a single copy plasmid greater than 100 Mdaltons in size so it is clearly necessary to transfer the uvrC to a more suitable vector for further study.

Other Cloning Studies With uvr Genes. Van Sluis and Pannekoek (8) have independently obtained plasmids carrying uvr genes. They have cloned a 1.5 Mdalton EcoRI fragment carrying the uvrB gene from a λbio uvrB transducing phage on the vector pMB9 to produce the recombinant plasmid pNP5. The expression of the uvrB gene depends on the genetic background. These workers also report that they have identified several plasmids with part of the uvrA gene and that the Clarke-Carbon plasmids pLC7-18 and pLC13-12 contain the entire uvrC gene. This finding is reasonable in that Clarke and Carbon (2) indicate that these two plasmids carry flaD which is adjacent to uvrC on the genetic map. We had also looked for uvrC on these 2 plasmids but did not find it. We could demonstrate the transfer of plasmids of about 7 and 10 Mdaltons respectively to a uvrC34 strain, but

the strain remained sensitive to UV. The trivial and most likely explanantion of this discrepancy is that the 2 laboratories are not working with identical plasmids although they are nominally the same. A more interesting possibility is that complementation of uvrC depends on specific alleles and strains that are used for the test.

Cloning of uvr Genes on λ. Two laboratories have obtained λ transducing phages carrying uvr genes by screening phage stocks in which restriction fragments were inserted in vitro. Seeberg, Steinum and Blingsmo (9) obtained a λp uvrC with the uvrC gene on a HindIII restriction fragment. Infection of cells with this phage results in the production of new proteins of 30,000 and 70,000 daltons, but the relation of these to the uvrC gene product is unclear. Auerbach and Howard-Flanders (10) have obtained λphages with the uvrA and the uvrC genes inserted on EcoRI restriction fragments.

Comparison of λ and Plasmids as Cloning Vectors. The results of McEntee (11) demonstrate that cloning of the recA gene on λ and on ColE1 is very useful in identifying and amplifying gene products. Cloning with λ and with plasmids have different advantages. A plasmid such as pBR322 allows much flexibility in the ease of manipulating cloned fragments and in minimizing the amount of vector DNA that is present in preparations of purified DNA. With λ it is possible to clone DNA fragments that would be lethal to the cell if cloned on a multicopy plasmid (12). It also offers the advantage that infection of lysogens prevents the expression of λ DNA while allowing the expression of cloned fragments with their own promoters. Cloning of genes on either λ or on plasmids results in amplification of the gene products although it is probable that greater amplification can be obtained with λ, particularly when appropriate λ mutants are used to delay or prevent lysis.

SUMMARY

The uvr genes of E. coli have recently been cloned in several laboratories. It is highly probable that the availability of these recombinant DNA's will lead to an early identification of the uvr gene products and will facilitate the understanding of their specific funetions in DNA repair.

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ENDONUCLEASE ACTIVITIES IN EXTRACTS OF MICROCOCCUS LUTEUS AGAINST GAMMA-IRRADIATED DNA

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ABSTRACT An endonuclease, specific for γ-irradiated DNA, was isolated from M. luteus and characterized. Per radiation induced strand break about 3 endonuclease sensitive sites are detected. The enzyme splits also DNA with apurinic sites as well as the alkali labile sites induced by γ-irradiation, but is inactive against UV-irradiated DNA.

INTRODUCTION

Four types of damage are predominant in DNA of γ-irradiated cells: Double-strand breaks, single-strand breaks, alkali labile lesions (1) and base damages. For repair of the latter two types of damage in the cell the DNA strand has to be split by a specific endonuclease to permit further enzymatic reactions. We recently isolated such an endonuclease from Micrococcus luteus (2). The enzyme seemed specific for γ-irradiated DNA. We report here that the enzyme preparation acts also on apurinic sites in DNA and on the alkali labile (presumably baseless) lesions induced by γ-irradiation.

METHODS

The isolation of the protein fractions from M. luteus has been described before (2). The endonuclease activity (in 20 mM tris buffer, pH 7.5) was measured analysing single-strand breaks in DNA by several methods: i) Activation of calf-thymus DNA as a primer for DNA polymerase I, where the amount of nucleotides incorporated corresponds to the number of nuclease induced strand breaks, ii) sedimentation of DNA in the analytical ultracentrifuge (3), iii) band forming sedimentation of PM2-DNA and separation of the supertwisted form from DNA with single-strand breaks (1), iv) alkaline sucrose gradient sedimentation of DNA isolated from Chinese hamster cells (CHO-cells) (4). γ-irradiation, UV-irradiation and preparation of DNA with apurinic sites were carried out as described (2).

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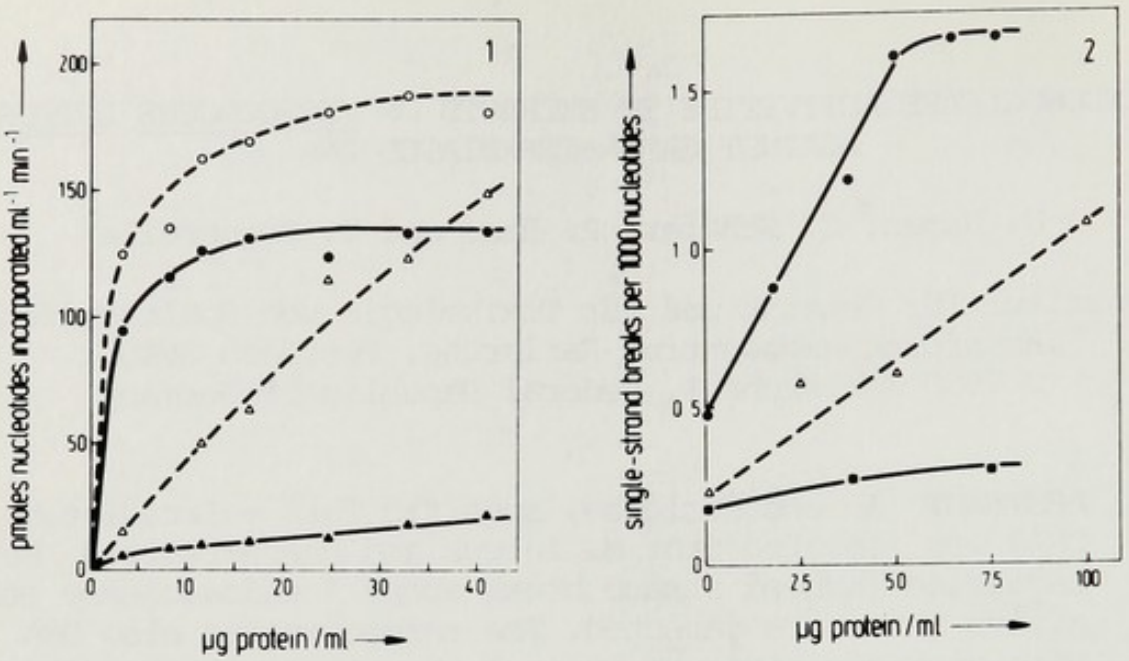

FIGURE 1. Action of the γ-endonuclease (prep. VIII) on various thymus DNA preparations (DNA polymerase test). ●——●, 500 rad in vitro (200 µg/ml in 0.01 M NaCl); o---o, 1 krad in vitro; ▲——▲, UV-irradiated DNA (5000 J/m^2) Δ---Δ, DNA with apurinic sites (2 sites per 1000 nucleotides)

FIGURE 2. Single-strand breaks in thymus DNA, induced by the γ-endonuclease (analytical ultracentrifugation) (prep.VII) ■ untreated DNA; ● 500 rad in vitro; Δ apurinic DNA

RESULTS

Gamma-irradiated DNA, UV-irradiated DNA, and apurinic DNA were incubated at 37° C with endonuclease (10 mM Mg^{++}), heated for 10 min at 60° C to inactivate the enzyme, and used as a subtrate of DNA polymerase I. Maximal activation by the enzyme of DNA with respect to primer activity depended on the radiation dose (Fig. 1). The kinetics of action on apurinic DNA showed a behaviour different from that on γ-irradiated DNA. There was only a small effect on UV-irradiated DNA. The actual number of strand breaks induced by endonuclease was determined by sedimentation analysis (3) (Fig. 2). There was an increase of strand breaks in γ-irradiated DNA up to a saturation value of about 3 nuclease-induced strand breaks per radiation-induced strand break. Untreated DNA was degraded only slightly.

The ionic conditions for endonuclease activity are described in table I. The activity against γ-irradiated DNA required Mg^{++}, whereas that against apurinic DNA was much less dependent on Mg^{++}. Further, the γ-endonuclease was inhibited by KCl but not the activity against apurinic sites.

TABLE I
IONIC CONDITIONS OF THE ENDONUCLEASE-ACTIVITY OF M. LUTEUS

Salt concentration	γ-irradiated DNA		apurinic DNA	
10 mM Mg^{++}		93[b]		per cent
7.5 mM "		96	"	"
5 mM "	100[a]	100	100[a]	" "
2 mM "		64	"	"
1 mM "		39	"	"
2 mM EDTA	22	19	74	" "
4 mM Mg^{++} + 10 mM KCl	112		98	" "
" + 20 "	101		96	" "
" + 50 "	89		120	" "
" + 100 "	46		110	" "

[a] activity determined with the DNA polymerase test (2)
[b] activity determined with the test on PM2 DNA (2)

The action of the endonuclease on PM2-DNA irradiated in 10 mM phosphate buffer, pH 6.5, is shown in Fig. 3. The sedimentation of the PM2-DNA was performed under neutral conditions as well as in alkali to detect the radiation induced alkali labile sites in addition to the single-strand breaks(ssb). After endonuclease treatment there was no difference of strand breaks under neutral or alkaline conditions. Evidently, the enzyme splits not only at the alkali stable endonuclease sensitive sites but also at the radiation-induced alkali-labile sites. In this particular experiment, 4 endonuclease sensitive sites (enss) per ssb were recognized by the enzyme one of which was an alkali labile site. The other sites can be considered to represent base damages. The ratio enss/ssb varied from 2.4 to 4.0 with various enzyme preparations.

The enzyme was also active against DNA irradiated in mammalian cells (Fig. 4). Endonuclease digestion reduced the sedimentation coefficient of DNA in alkaline sucrose gradients.

DISCUSSION

The endonuclease we isolated from M. luteus showed two different activities: one acts on γ-irradiated DNA and depends on the presence of Mg^{++}. About 3 endonuclease sensitive sites are split by this enzyme per radiation induced strand break. The other activity, independent of Mg^{++}, splits apurinic sites in DNA; it is active also against the radiation induced alkali labile sites. This activity may correspond to the endonuclease for apurinic DNA isolated by Tomilin et al. (5) from M. luteus.

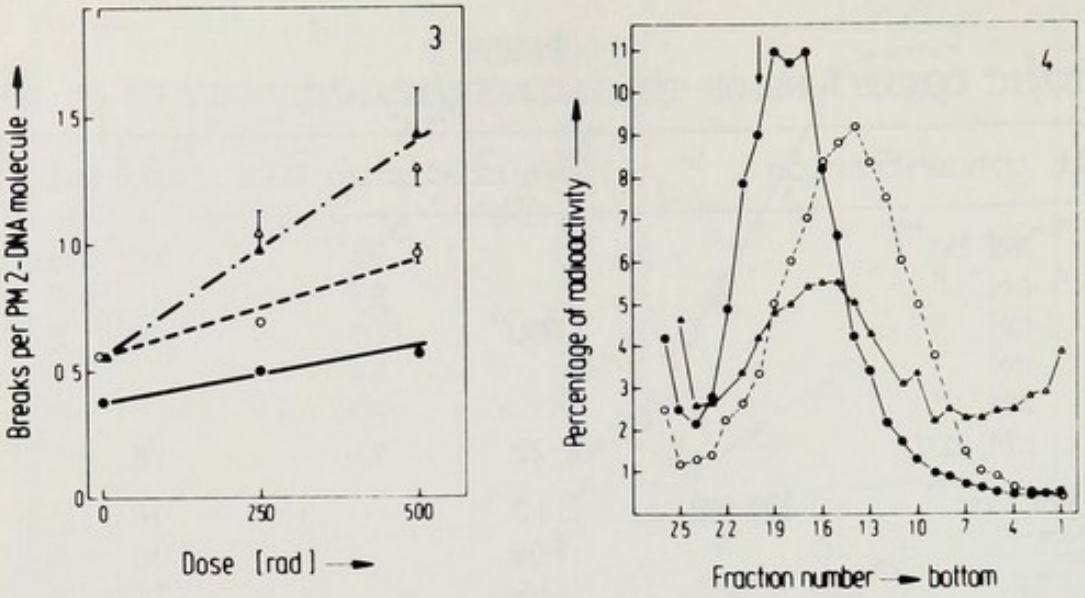

FIGURE 3. Formation of strand breaks in PM2 DNA by γ-irradiation in vitro and endonuclease treatment. ●——●, γ-irradiation, neutral sedimentation; o---o, γ-irradiation, alkaline sedimentation; ▲△-.-.-△▲, γ-irradiation + endonuclease treatment, neutral (▲) or alkaline (△) sedimentation.

FIGURE 4. Alkaline sucrose gradient sedimentation profiles of DNA, isolated from CHO cells after 5 krad (90 min at 32000 rev./min). ●, DNA, untreated; o, DNA, incubated with γ-endonuclease in presence of 2 mM EDTA; △, DNA, incubated with γ-endonuclease in presence of 6 mM $MgCl_2$. ↓ Position of DNA from coli phage T1.

The γ-endonuclease activity detected shows properties similar to that isolated by Carrier and Setlow (6). Our endonuclease preparation, however, is inactive against UV-irradiated DNA.

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WORKSHOP SUMMARY: EXCISION REPAIR PATHWAYS IN BACTERIA

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Superficially, excision repair, a mechanism used by most living organisms to accomplish the dark repair of damaged DNA, appears to be quite simple. However, the situation is complicated by the fact that *Escherichia coli*, and perhaps other organisms as well, maintains several enzymes with similar *in vitro* function. For example, there are at least three DNA polymerases in *E. coli* capable of performing the resynthesis step of excision repair, and at least three 5'→3' exonucleases capable of excising pyrimidine dimers from DNA *in vitro* (1,2). Moreover, a number of mutations of *E. coli* are known to affect excision repair although the exact enzymatic deficiency associated with these mutations has, in several cases, yet to be elucidated. Thus, when the biochemical details of excision repair are considered there are numerous pathways by which excision repair can proceed, and some of these appear to be quite complex.

Kendric Smith and his co-workers have delineated a number of repair pathways in *E. coli* (3,4) some of which are dependent upon growth medium and require protein synthesis for completion. Studies with mutants of *E. coli* demonstrate the requirement for the products of such genes as *uvr*D, *rec*A, *rec*B, *lex*A, *pol*B, *pol*C, and *lig* in the growth-medium-dependent pathways. Of particular interest was the claim (K. C. Smith *et al.*, this volume) that DNA polymerase II is primarily responsible for the generation of long patches of resynthesis during excision repair. Earlier studies (5,6) have demonstrated that in *pol*A strains either DNA polymerase II or III can perform repair resynthesis. Since *pol*A mutants maintain the 5'→3' exonuclease associated with DNA polymerase I (7), Smith's result suggests that the presence of this exonuclease, without the DNA synthetic function of DNA polymerase I, may alter the incision into a form suitable for activity by either DNA polymerase II or III. Also since *pol*B mutants (deficient in DNA polymerase II) do not show altered viability or ultraviolet (UV) sensitivity (8) this deficiency in long-patch repair can apparently be compensated for by other repair pathways.

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Additional data accumulated by Smith and his associates show that although *uvr*D and *rec*B mutants show UV-induced mutation the double mutant *uvr*D *rec*B does not produce mutations after exposure to UV. This suggests that exonuclease V and the *uvr*D gene product may control alternate branches of an error-prone DNA repair pathway.

Cooper and Hanawalt have discovered a heterogeneity in the size of the patch of DNA resynthesized during repair (9). In *pol*A and *pol*A*ex* strains longer repair patches appear to dominate (10,11). Data presented by P. K. Cooper (Cooper and Hunt, this volume) indicate that inhibition of protein synthesis during repair reduces the amount of repair resynthesis. However, prior exposure of exponentially growing cells to a sublethal dose of UV followed by a period of protein synthesis removes the requirement for protein synthesis during the resynthesis step. Additional controls demonstrate that it is the long-patch component of repair resynthesis mediated by DNA polymerases II and III that is primarily affected by the protein synthesis requirement. These results suggest that an element of repair resynthesis may be inducible and call attention to the possibility that under certain conditions excision repair may result in mutation.

Cooper has also examined the excision of pyrimidine dimers after UV irradiation of *pol*A strains with temperature sensitive mutations in *rec*B and *rec*C to control the postirradiation DNA degradation apparent in previous studies with *pol*A (12) and *pol*A*ex* (11,13) strains. Her data indicate that although *pol*A*ex* *rec*B*ts* *rec*C*ts* mutants (deficient in the 5'→3' exonuclease of DNA polymerase I) are slower than wild type at dimer excision the *pol*A *rec*B*ts* *rec*C*ts* mutants (deficient in the DNA synthetic activity of DNA polymerase I) are more deficient at excision than either wild-type or *pol*A*ex* *rec*B*ts* *rec*C*ts* strains. This result suggests that concerted action by the DNA synthetic function and the 5'→3' exonuclease of DNA polymerase I is necessary to achieve the maximum rate of dimer excision. These observations agree well with *in vitro* studies (14) that showed an increased level of dimer excision by DNA polymerase I when DNA synthesis occurred. Cooper has found that a *pol*A *pol*C strain, deficient in both DNA polymerase I and III, is acutely deficient in its ability to cope with dimers. This result is complicated by lack of information as to whether the 5'→3' exonuclease of DNA polymerase III is affected in the *pol*C1026 mutant used and by this mutant's extreme postirradiation DNA degradation. However, the observed excision deficiency is compatible with the notion that a close coupling between excision and resynthesis is essential for efficient excision repair.

In vivo measurements of pyrimidine dimer excision in an isogenic series of strains deficient in exonuclease V, the 5'→3'

exonuclease of DNA polymerase I, and exonuclease VII show that, at least in the absence of exonuclease V, exonuclease VII and the 5'→3' exonuclease of DNA polymerase I can apparently substitute for one another in removing dimers from DNA (Masker and Chase, this volume).

Studies of excision repair in toluene-treated *E. coli* have shown that incision at the site of pyrimidine dimers can occur only when ATP is present (15). In wild-type cells only the incision step requires ATP (16). However, *pol*A strains show an additional ATP requirement for repair resynthesis (17). Ruth Ben-Ishai and collaborators (Ben-Ishai *et al.*, this volume) have used toluene-treated cells to make measurements of the amount of repair resynthesis performed by wild-type *E. coli*. By measuring dimer excision and deoxyribonucleoside triphosphate incorporation into DNA Ben-Ishai obtained a value of 13-16 nucleotides incorporated per pyrimidine dimer removed. This agrees well with previously reported values obtained with an *in vitro* repair system capable of restoring the biological activity of damaged DNA (18,19). Data presented by Ben-Ishai indicates that in *pol*A strains treated with toluene a component of excision repair is inducible.

Ben-Ishai and co-workers have also examined the closure of postreplicational repair gaps in irradiated DNA of toluene-treated cells. It was found that by pretreating exponentially growing cells with a low dose of UV and allowing a period of protein synthesis before toluene treatment about 20% of the gaps in irradiated DNA could be repaired. These data suggest the presence of an inducible form of postreplicational repair in a quasi *in vitro* system.

Excision mechanisms also play a role in the repair of cross-links in DNA. Removal of psoralen cross-links occurs through a scheme involving incision, excision, and recombination. Studies by R. Cole and his associates (20,21, Cole *et al.*, this volume) have shown that the initial incision event requires the *uvr*A, *uvr*B, and *uvr*C gene products of *E. coli*. The incision step also requires Mg^{++} as well as ATP (21). Excision of the cross-link appears to require the 5'→3' exonuclease of DNA polymerase I as well as the *uvr*D gene product. The *rec*F gene product is required to open a large (1000 nucleotide) single-strand region subsequent to cross-link removal. Finally, repair is completed by recombinational events mediated by the *rec*A recombination pathway. Thus, completion of repair after cross-link removal provides a suitable assay for recombination. An examination of cross-link removal using mutants temperature-sensitive in *rec*A has led to the development of a model for genetic recombination (22). This model predicts single occurring, successive recombinational events taking place progressively around the genome. Measurements of parameters such as the kinetics of repair after cross-link

removal, cell survival, and DNA molecular weights during the repair process agree with this model for recombination.

The discussion above emphasizes the complexity of excision repair mechanisms in bacteria and underscores the need for further experimentation in this area. As of yet the incision step at the site of pyrimidine dimers is not completely understood. Since the nature and location of the incision probably determines the type of substrate available to excision and resynthesis enzymes, the incision event itself may dictate which of the available excision repair pathways is followed. Thus, understanding the exact role of the *uvr*A, *uvr*B, and *uvr*C gene products is essential. Also, there is a clear need for additional experimentation with mutants such as *uvr*D, *uvr*E, *rec*L and *mfd*. Apparently protein synthesis is required for completion of excision repair by certain pathways, but as of yet the identity of the essential protein(s) remains obscure. There appears to be general agreement that the 5'→3' exonuclease of DNA polymerase I is important in dimer removal although the degree of excision deficiency associated with *polAex* mutants may depend on the experimental procedure or the particular *E. coli* strains employed. Also, although there is some controversy concerning the difficulties caused by postirradiation DNA degradation, experiments involving high levels of DNA degradation should be interpreted cautiously. Thus, the excision repair scheme involving incision, excision, resynthesis, and ligation is almost certainly well founded, and considerable progress has been made toward understanding the details of individual steps. Much additional effort directed toward understanding the order of events, the intermediate DNA structure produced, and particularly, the detailed biochemistry of the enzymes involved is needed before the intricacies of excision repair are fully realized.

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EXCISION REPAIR AND MUTAGENESIS ARE COMPLEX PROCESSES[1]

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ABSTRACT Excision repair in *Escherichia coli* has been divided into two major pathways: a growth medium-independent, *polA*$^+$-dependent pathway, and a growth medium, *recA*$^+$, *recB*$^+$, *lexA*$^+$, *polC*$^+$, *uvrD*$^+$-dependent pathway. The former pathway handles most of the UV radiation-induced lesions, and produces short patches of repair replication. The latter pathway can be subdivided into two branches: a *uvrD*$^+$-dependent branch, and a *uvrD*$^+$-independent branch. Long patch repair replication appears to occur via this latter branch; it is *recA*$^+$, *lexA*$^+$ and *polB*$^+$-dependent, and is inhibited in buffer or by chloramphenicol (CAP). The *uvrD*$^+$ gene product can also function independently of *recA*$^+$ if UV-irradiated cells are held in buffer for a time before plating. Thus, liquid holding recovery (LHR), an excision repair process that is observed in *recA* strains, is blocked by a *uvrD* mutation, but is enhanced by *lexA* and *recB* mutations. UV radiation-induced mutations are produced by excision repair as well as postreplication repair. In either case, they appear to occur via two subbranches of these repair systems; one controlled by *recB*$^+$ and the other by *uvrD*$^+$.

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MULTIPLE PATHWAYS OF EXCISION REPAIR

Excision repair has been divided into two major pathways (Fig. 1). One pathway is growth medium-independent (1), and *polA*$^+$-dependent (1-3). The second pathway is growth medium, *recA*$^+$, *recB*$^+$, *lexA*$^+$, and *polC*$^+$(1) dependent, and partially *uvrD*$^+$(4) dependent, and is inhibited by dinitrophenol (5), CAP (1), and by impurities in most brands of agar (6). Both pathways are *lig*$^+$-dependent (7).

The first pathway handles most of the UV radiation-induced lesions (1), and produces short patches of repair replication (8,9). The second pathway can be divided into two branches: a *uvrD*$^+$-dependent branch, and a *uvrD*$^+$-independent branch (4). Long patch repair replication appears to

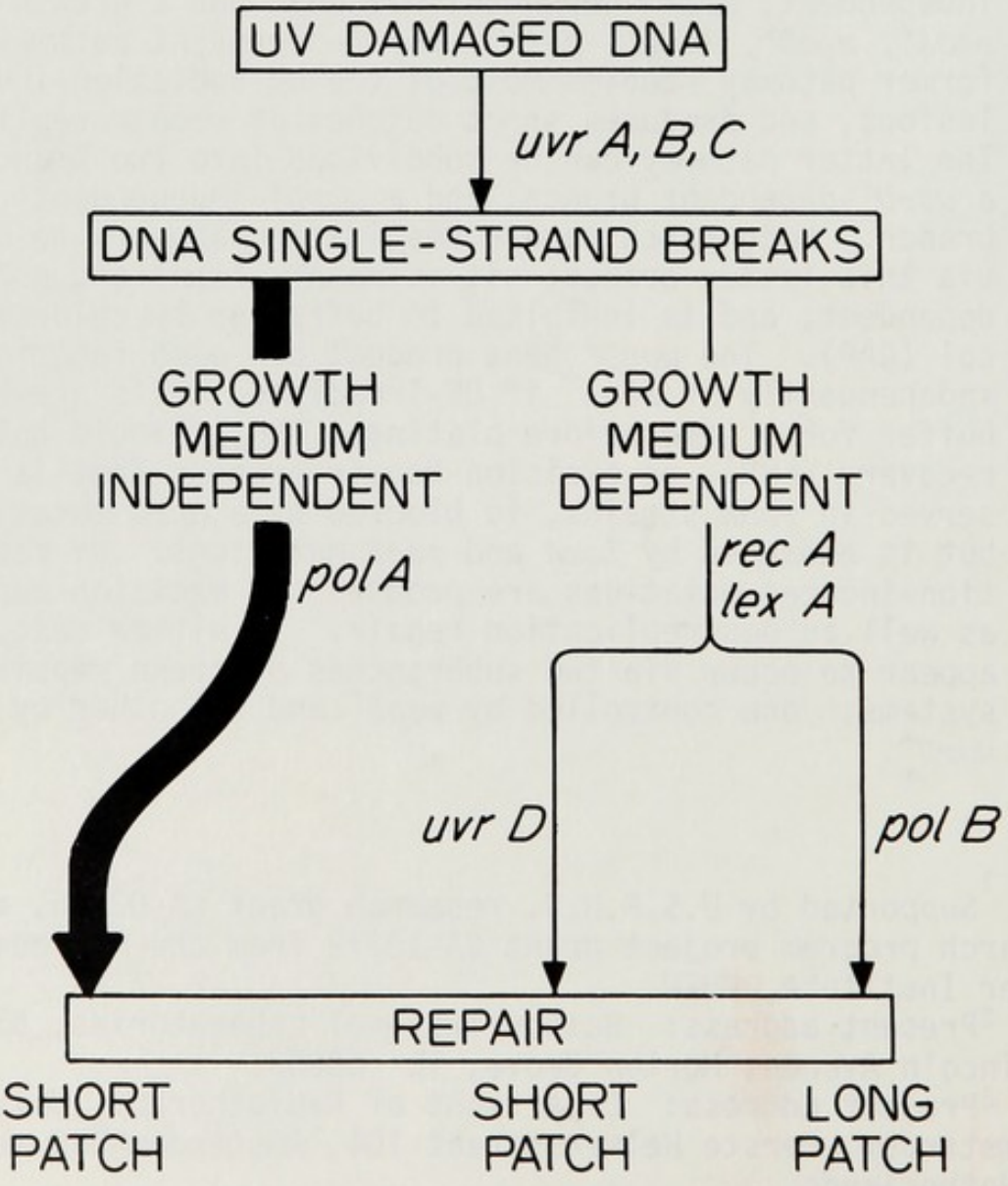

Figure 1. The multiple pathways of excision repair.

occur via this latter branch (9); it is inhibited in buffer or by CAP (9), is *recA*$^+$ (8) and *lexA*$^+$(9) dependent, and shows an absolute requirement for *polB*$^+$ in *polA*$^+$ strains (9). Therefore, since a *polB* mutation has little effect on survival (10) or mutagenesis (9) after UV irradiation, long patch excision repair appears to play no significant biological role after UV irradiation. However, we are searching for situations where it may play a significant biological role (e.g., after chemical damage to cellular DNA).

LIQUID HOLDING RECOVERY

Liquid holding recovery (LHR) is an excision repair process that is observed in *recA* strains of *E. coli* K-12 (11). Operationally, UV-irradiated cells are held in buffer for periods of up to 24 h before plating on growth medium. The recovery of viability that occurs during this holding period is blocked by a mutation at *uvrD*, but is enhanced by mutations at *recB* or *lexA* (12). Although *uvrD*$^+$ functions show an absolute requirement for *recA*$^+$ functions when UV-irradiated cells are maintained in growth medium (i.e., a *uvrD* mutation does not sensitize a *recA* strain), the *uvrD*$^+$ gene product can function in the absence of *recA*$^+$ functions when UV-irradiated cells are held in buffer before plating. Thus, under the conditions of LHR, a *uvrD* mutation sensitizes a *recA* strain to UV radiation.

UV RADIATION MUTAGENESIS

A *recA* (13) or a *lexA* (14) mutation blocks UV radiation mutagenesis. The fixation of UV radiation-induced mutations is blocked in a *uvrA* strain by a postirradiation treatment with CAP (15). Both the *recB*$^+$ and *uvrD*$^+$ gene functions are required for the CAP-inhibitable pathway of postreplication repair (16).

A *recB* mutation has a small effect on the expression of UV radiation-induced mutations, while a *uvrD* mutation has a larger effect (17). Since individually a *recB* or a *uvrD* mutation does not completely eliminate UV radiation mutagenesis, and since both gene products are involved in the CAP-inhibitable (i.e., mutagenic) branch of postreplication repair (15,16) and of excision repair (1,4), we postulated that the *recB*$^+$ and *uvrD*$^+$ genes may function on separate pathways of repair that affect mutagenesis. Therefore, we have tested strains carrying both a *recB* and a *uvrD* mutation for UV radiation mutagenesis.

Consistent with our hypothesis, our *recB uvrD* and *uvrB recB uvrD* strains show no UV radiation mutagenesis when screened for *leuB* (missense mutation)⟶Leu$^+$, or for *lacZ* (amber nonsense mutation)⟶Lac$^+$. Therefore, the *recB*$^+$ and *uvrD*$^+$ genes must control two separate error-prone repair pathways for producing UV radiation-induced mutations. When both pathways are blocked, no UV radiation-induced mutations are detectable (17).

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UVRD, *UVRE* AND *RECL* REPRESENT A SINGLE GENE[1]

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ABSTRACT A series of isogenic strains carrying either *uvrD3*, *uvrD101*, *uvrE4*, *uvrE100*, *uvrE502* or *recL152* were analyzed for sensitivity to ultraviolet light, increased spontaneous mutation frequency and ability to carry out host cell reactivation. All strains showed similar levels of UV sensitivity and ability to carry out host cell reactivation. *uvrD* and *uvrE* mutants exhibited significant increases in spontaneous mutation frequency compared to wild type or *recL152* strains. All the alleles were recessive in transconjugants carrying a wild type F' plasmid. F' plasmids carrying either *uvrE502* or *recL152* failed to complement any of the above alleles but did complement *rep-3*. Strains carrying *polA12* and either *recL152*, *uvrD3*, *uvrD101*, *uvrE4* or *uvrE502* were conditionally lethal for growth and showed altered recombination properties at 37°.

INTRODUCTION

Within the past ten years a number of mutations have been identified which affect UV sensitivity, host cell reactivation, spontaneous mutation frequency and genetic recombination and which map between *ilv* and *metE* on the *E. coli* chromosome. UV sensitive mutants isolated by Ogawa et al. were designated *uvrD*(1). Based on complementation studies in the original mutant it was suggested that *uvrD3* was dominant. Subsequently Siegel isolated mutants of *E. coli* showing increased spontaneous mutation frequency and increased sensitivity to UV, which were called *mutU*(2). Smirnov and Skavronskaya also isolated mutants of similar properties which were called *uvrE*(3). Finally, Horii and Clark identified the *recL* gene following mutagenesis of a recombination proficient strain of *E. coli* which was *recB21 recC22 sbcB15*(4).

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Since Smirnov has indicated that *uvrE502* and *mutU4* are alleles of the same gene (5), the *mutU* designation has been dropped. Additionally, Smirnov and Abdukhalykova have obtained evidence to suggest that the *uvrE502* and *recL152* alleles are related (6).

Employing a series of isogenic strains, the properties of *uvrD*, *uvrE* and *recL* mutants have been reexamined. In addition, fine structure mapping of the *ilv*-*metE* region of the *E. coli* chromosome has been carried out. The results of some of these experiments are described below.

RESULTS

Properties of *uvrD*, *uvrE* and *recL* Single Mutants. Strains carrying either *uvrD3*, *uvrD101*, *uvrE4*, *uvrE100*, *uvrE502* or *recL152* were analyzed for UV sensitivity, increased spontaneous mutation frequency and ability to carry out host cell reactivation. All of the mutants showed similar levels of UV sensitivity and ability to repair UV irradiated bacteriophage lambda. As shown in Table 1, *uvrD* and *uvrE* mutants exhibited increased spontaneous mutation frequencies compared to wild type and *recL152* strains.

All of the alleles were tested for dominance by construction of transconjugants carrying a F' plasmid extending from *ilv* through *metE*. In contrast to the results of Ogawa et al. (1) both *uvrD3* and *uvrD101* were recessive to *uvrD*$^+$ on the basis of host cell reactivation and UV sensitivity. The *recL* and *uvrE* loci were also recessive. In each case the mutant allele was rescued from the transconjugant by transduction with P1*vir*.

TABLE 1
COMPARISON OF MUTATION FREQUENCIES

Strain	Frequency of Mutation x 10^8	
	ilv	*his*
SK723 (wild type	8.8 ± 1.7	7.8 ± 1.2
SK719 (*recL152*)	25.4 ± 3.1	7.3 ± 0.7
SK728 (*uvrD3*)	450 ± 55	64 ± 13
SK774 (*uvrD101*)	131 ± 12	29 ± 2
SK726 (*uvrE100*)	325 ± 40	31 ± 4

Figures represent an average of 20-30 independent cultures grown overnight and 0.1 ml from each plated out on appropriate selective media.

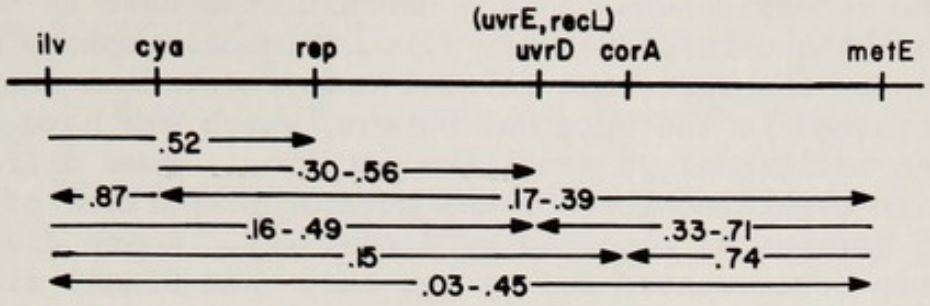

Figure 1. Linkage map of the *ilv-metE* region.

Fine structure Analysis of the *ilv-metE* Region. A series of three factor crosses involving *ilv*, *cya*, *metA*, *corA* (7), *rep*, *uvrD*, *recL* and *uvrE* were carried out. The linkages are outlined in Figure 1. Double mutants containing any combination of *uvrD*, *uvrE* or *recL* alleles were not detected. Additionally, wild type recombinants were obtained in unusually low yields (<1%) in these crosses, indicating a very close linkage relationship. In contrast wild type recombinants occurred at expected levels in crosses involving *rep-3* and the above three loci. F' plasmids were obtained which either terminated before *uvrD*, *uvrE* and *recL* or which complemented all three loci, again suggesting close linkage.

F' primes carrying either *uvrE502*, *uvrD3* or *recL152* failed to complement strains carrying mutations in *uvrD*, *uvrE* or *recL*. The *rep-3* allele was complemented by these F' plasmids. Each F' plasmid was shown to carry the marker in question by P1*vir* transduction.

Properties of Multiple Mutants. Mattern and Houtman (8) and Siegel (9) had obtained evidence to indicate that *uvrE* and *polA* mutations were incompatible. A series of strains carrying *polA12* (temperature sensitive polymerizing activity) or *polA107* (deficient in the 5'-3' exonuclease) and mutations in *uvrD*, *uvrE* or *recL* were constructed. All the strains containing *polA12* were conditionally lethal for growth, while the multiple mutants obtained with *polA107* were viable at all temperatures. The *polA12* multiple mutants showed reduced levels of genetic recombination at 37° when measured by the assay of Zieg and Kushner (10).

DISCUSSION

Many of these findings are in agreement with work of

others (5,6,8,9, A. J. Clark, personal communication). However, the recessiveness of *uvrD* demonstrated here is not in agreement with earlier results (1). This discrepancy may be explained by the fact that initial complementation analyses were performed in the original mutant, which may have contained an additional UV sensitive mutation. Some differences also exist regarding the Hcr and mutator properties of *uvrD* and *uvrE* strains as reported by Siegel (2). These discrepancies may be accounted for by the fact that Siegel did not compare the *uvrD3* and *uvrE4* alleles in the same genetic background.

The results presented above suggest that *uvrD*, *uvrE* and *recL* represent a single gene. It is suggested that all these alleles be designated as *uvrD*. From data obtained from nuclease deficient strains of *E. coli*, it appears that the *uvrD* protein may serve to regulate either DNA polymerase I or DNA polymerase III.

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ALTERNATIVE PATHWAYS FOR EXCISION AND RESYNTHESIS IN *ESCHERICHIA COLI*: DNA POLYMERASE III ROLE?[1]

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ABSTRACT Measurements of *in vivo* rates of dimer excision in mutants of *Escherichia coli* suggest that polymerizing activity of DNA polymerase I is more important for efficient excision than any one 5'-3' exonuclease activity and that polymerase III can promote excision in the absence of polymerase I. Studies of the resynthesis step of excision repair show that the long patch component of repair synthesis, which is presumably due to polymerase II and/or III, is UV-inducible and requires protein synthesis.

INTRODUCTION

DNA polymerase I of *Escherichia coli* has the *in vitro* capacity to simultaneously remove pyrimidine dimers from UV-irradiated DNA and resynthesize the excised region (1). Mutants deficient in either the polymerizing activity or the 5'-3' exonuclease activity of pol I show increased UV sensitivity but dimers are efficiently excised following low UV doses and repair synthesis is increased (2). Apparently enzymes alternative to pol I operate in both excision and resynthesis. Repair synthesis in *polA* mutants can be performed by either polymerase II or III (3), and presumably either or both of these enzymes are involved in the long patch repair which occurs more frequently in *polA* (4,5). Earlier work has shown that long patch repair appears to require the *recA*$^+$ genotype (5), and a pol I independent pathway of repair of excision gaps has a similar requirement for *recA*$^+$, in addition to *lexA*$^+$ and protein synthesis (6). It has been proposed that inducible error-prone repair occurs both during post-replication repair and during excision repair at sites of closely-spaced lesions (7). We have tested the hypothesis that long patch repair may be a manifestation of the SOS repair system operating at excision gaps by examining the effects of post-UV inhibition of protein synthesis and of pre-

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irradiation on repair synthesis.

Earlier studies of dimer excision in pol I-deficient mutants showed that for low UV doses excision was nearly as efficient as in wild-type strains, but interpretation of an apparent decrease in excision ability following higher doses was complicated by extensive post-irradiation DNA degradation in both 5'-exonuclease deficient and polymerase-deficient mutants (2). To eliminate this difficulty of interpretation we have measured excision in similar strains additionally deficient in exonuclease V. Like pol I, DNA polymerase III has an associated 5'-3' exonuclease activity which is capable of *in vitro* dimer excision. (8) We have examined a *polApolC* double mutant for a possible contribution of a pol III activity to excision *in vivo*.

RESULTS

We constructed the strain PC16L (*recB270recC271polAex2*) by P1 transduction of the temperature sensitive *recB* and *recC* mutations (9) into the pol I 5'-exonuclease deficient strain RS5069 (2); this proved to be a lethal combination, and strain PC16L is inviable at 43°. A similar strain has been independently constructed by Masker and Chase (this volume). We obtained strain SK211 (*recB270recC271polA1*), which is also conditionally lethal, from Kushner (9). These triple mutant strains are much more UV-sensitive at 32° than their parents, and post-irradiation DNA degradation is considerably reduced at 43° compared to the *polAex2* and *polA1* single mutants.

Kinetics of thymine dimer excision at 43° were measured as previously described (2) following irradiation for strains W3110 (wild type, *thy*$^-$), RS5069 (*polAex2 thy*$^-$), PC16L (*recB270recC271polAex2 thy*$^-$), and HS434 (*polA1polC1026 thy*$^-$, ref.3). Table I gives the per cent of induced dimers remaining in acid-precipitable DNA after 45 min incubation; in most cases this is close to a plateau value for extent of removal. There are no significant differences in the apparent excision ability measured for PC16L compared to RS5069 or for SK211 compared to JG138. Thus, extensive DNA degradation by exonuclease V does not appear to affect the measurement of preferential dimer excision in acid-precipitable DNA (cf. ref. 2).

Deficiency in the pol I 5'-exonuclease results in a slight decrease in excision (2); the decrease is most pronounced at higher doses (Table I). However, deficiency in pol I polymerizing activity produces an even greater defect in excision, and after doses of 60 J/m^2 or greater, only half or less of the dimers are excised by JG138 or SK211, compared to essentially complete removal by wild type. We conclude that pol I synthetic activity is more stringently required for efficient excision than is the 5'-exonuclease activity, perhaps

TABLE I

DIMER EXCISION

J/m^2, Incident	$\frac{\hat{TT}}{T}$ x 100, 0 min	% Initial $\frac{\hat{TT}}{T}$ remaining after 45', 43° W3110	RS5069	PC16L	JG138	SK211	HS434
25	0.075	15	10		25	25	65
45	0.14		20	20	35	45	70
60	0.18	20	25	30	45	45	75
80	0.24	15	35		60	65	80
100	0.31	15	40	50	70		90

as a result of the existence of alternative excision nucleases (Masker and Chase, this volume). It was therefore of interest to examine the dimer excision capacity of a polA1 mutant additionally defective in polymerase III. We find that excision proceeds to a very limited extent in the polA$^-$ polC$^-$ double mutant HS434 even at low doses (Table I) and conclude that an activity of polymerase III is required for in vivo dimer excision in the absence of pol I polymerizing activity. These results are qualified by the fact that post-UV DNA degradation is even more pronounced in HS434 than in JG138 but as noted above we believe that extensive degradation probably does not interfere with dimer excision determinations.

We examined the resynthesis step of excision repair under conditions of post-UV protein synthesis inhibition in the repair proficient strain W3110 (Table II) by density labelling and sedimentation analysis as previously described (4). Cells were prelabelled with ^{14}C-Thy at 32°, prewarmed at 43°, irradiated with 40 J/m^2 UV, and incubated for 45 min at 43° with ^{3}H-BrUra repair label and growth supplements, with or without 100 ug/ml chloramphenicol (CAP). Repair was assayed at 43° to allow direct comparison of these results with those for temperature sensitive strains in other experiments. Repair synthesis (the ratio of ^{3}H to ^{14}C in isolated parental density DNA) is reduced by about 45% by CAP after this dose and is further reduced if CAP is also added to the prelabelling medium 30 min prior to irradiation (samples 2, 3, and 5 of Table II). The proportion of repair synthesis which requires protein synthesis varies with dose; after 20 J/m^2 in other experiments the reduction by CAP is reproducibly 20-30%. The requirement for post-UV protein synthesis can be relieved if the cells are pre-irradiated and allowed 30 min of growth prior to administering a second dose and assaying repair synthesis (samples 6 and 7); the pre-irradiation is effective only if protein synthesis is allowed between the two doses (sample 9).

TABLE II

REPAIR SYNTHESIS

Sample	Treatment	Repair
1	no UV, no CAP	18.9
4	+CAP, no UV, +CAP	6.8
2	40 J/m^2, -CAP	118.2
3	40 J/m^2, +CAP	64.8
5	+CAP, 40 J/m^2, +CAP	47.3
8	20 J/m^2, -CAP	28.1
6	20 J/m^2 -CAP; 40 J/m^2 -CAP	84.1
7	20 J/m^2 -CAP; 40 J/m^2 +CAP	84.9
9	20 J/m^2 +CAP; 40 J/m^2 +CAP	42.4

We analyzed the distribution of repair patch sizes in the presence and absence of protein synthesis by shearing the repaired parental density DNA from the above experiment and banding in alkaline CsCl essentially as previously described (4), except that the fragment size was about 250 nucleotides and sedimentation was in a swinging bucket rotor. As shown in Figure 1, the "long patches" (density-shifted ^3H repair label) which are prominent when protein synthesis is allowed (45% of total ^3H in sample 2) are preferentially reduced by CAP (samples 3 and 5). The inhibition of long patch synthesis by CAP is prevented by pre-irradiation (samples 6 and 7 both have long patches containing 45% of total ^3H) but not when CAP is present between the two irradiations (sample 9). Identical results were obtained when the experiment was performed with a dnaB mutant at the restrictive temperature, thus eliminating any possibility that we might be confusing long repair patches with short stretches of replication. We conclude that long patch repair synthesis is an inducible phenomenon. This interpretation would predict that a greater proportion of the repair synthesis in a polA1 mutant which performs increased large patch repair would require new protein synthesis; we have found this to be true for two different polA1 strains.

DISCUSSION

Our studies of in vivo dimer excision in a variety of mutant strains indicate that excision proceeds efficiently only when it is coupled with the repair resynthesis step, since

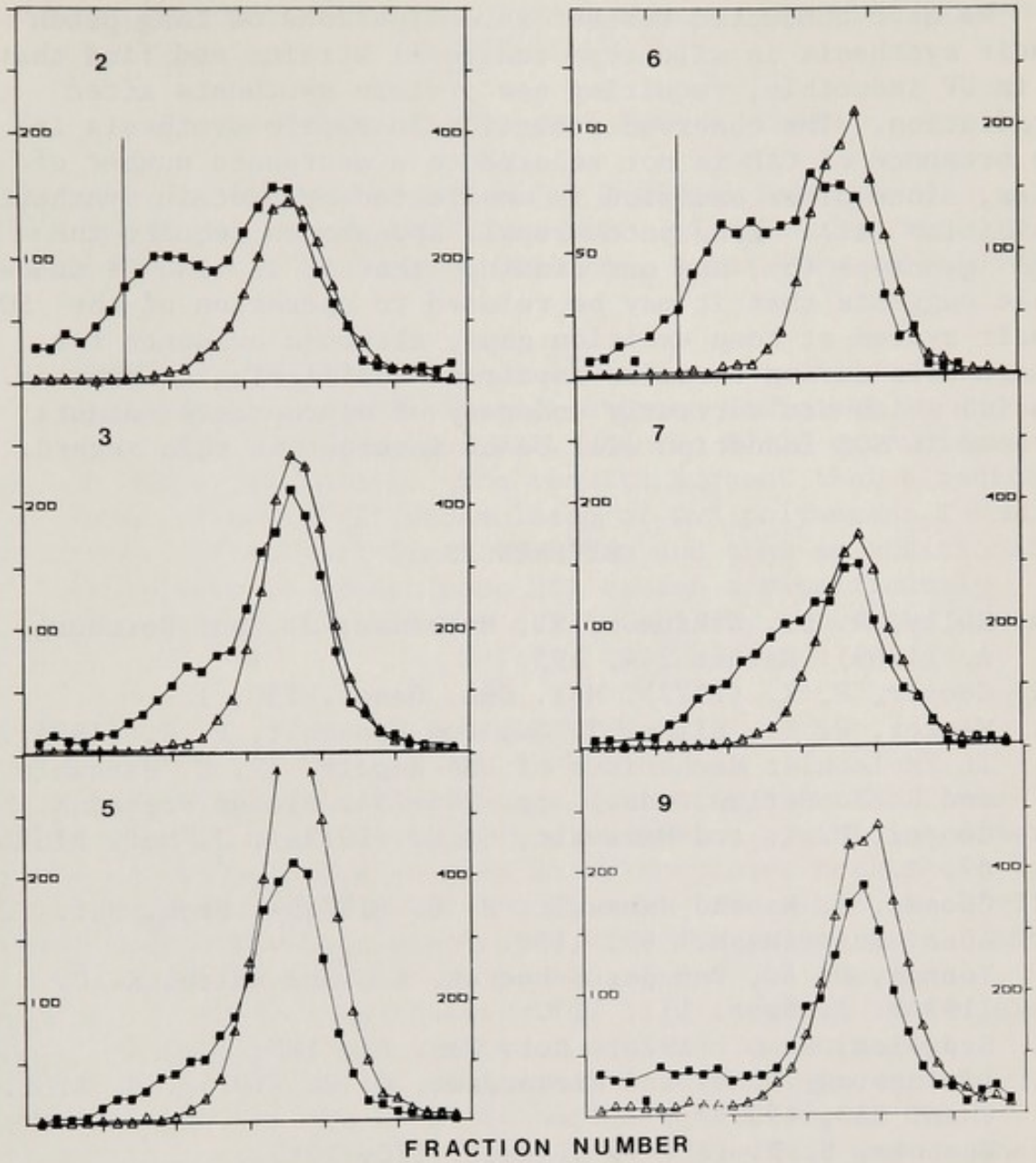

FIGURE 1. Isolated parental density DNA from indicated samples from Table II, sonicated and banded in alkaline CsCl, SW50.1 rotor. Line marks position of fully BrUra-substituted strand. △ = ^{14}C-thy cpm, right axis; ■ = ^{3}H-BrUra cpm, left axis.

polA1 mutants are more defective in excision than are the 5'-exonuclease deficient mutants. Direct evidence for coupling of excision and resynthesis by polymerase I has been obtained in vitro (10). The extreme excision defect in HS434 may mean that polymerase III can provide a necessary "driving" synthetic capacity for excision in the absence of pol I, although this interpretation is qualified by the fact that it is not known whether the pol III 5'-exonuclease activity is also deficient in this mutant.

We have conducted further investigations of long patch repair synthesis in wild type and *polA1* strains and find that it is UV inducible, requiring new protein synthesis after irradiation. The observed reduction in repair synthesis in the presence of CAP is not related to a decreased number of sites, since dimer excision is unaffected by protein synthesis inhibition (11). Long patch repair appears to require the *recA*$^+$ genotype (5), and our finding that it is also UV inducible suggests that it may be related to operation of the SOS repair system at some excision gaps, although evidence for mutagenesis during excision repair is conflicting at present. Studies which are currently underway of appropriate mutants altered in SOS induction will be of interest in this regard.

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PYRIMIDINE DIMER EXCISION IN EXONUCLEASE DEFICIENT MUTANTS OF *ESCHERICHIA COLI*[1]

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ABSTRACT The rate of pyrimidine dimer excision has been measured in *Escherichia coli* strains deficient in exonuclease V, exonuclease VII, and the 5'→3' exonuclease of DNA polymerase I. The results suggest that a reduced level of the 5'→3' exonuclease of DNA polymerase I diminishes the rate of dimer excision and that an additional deficiency in exonuclease VII causes a significantly greater reduction in the cell's ability to remove dimers.

INTRODUCTION

Three 5'→3' exonucleases are currently known in *Escherichia coli* which may be capable of removing pyrimidine dimers in the excision repair process since they have been shown to excise pyrimidine dimers *in vitro* (1,2,3). These are the 5'→3' exonuclease activities associated with DNA polymerases I and III and exonuclease VII. We have extended our previous studies (4) on the exonucleases of *E. coli* involved in excision repair by examining the effect of combined mutations in *recB,C*, *xseA*, and *polAex* (deficient in exonuclease V, exonuclease VII, and the 5'→3' exonuclease activity of DNA polymerase I, respectively) (5,6,7) on UV survival and on the rate and extent of dimer excision. Our earlier study (4) demonstrated that mutants deficient in exonuclease VII and the 5'→3' exonuclease activity of DNA polymerase I are nearly as efficient as wild-type strains at excising dimers produced by up to 40 J/m^2 UV. At higher doses strains containing only the *polA546ex* mutation and the *polA546ex xseA7* mutations show reduced ability to excise dimers; however, the interpretation of dimer excision data at these doses is complicated by extreme postirradiation DNA degradation in these strains. We

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have attempted to reduce postirradiation DNA degradation by reducing the level of exonuclease V (the *recB,C* gene product) in order to evaluate the apparent deficiency in dimer excision at high UV doses observed in the *polA546ex* single mutant and the *polA546ex xseA7* double mutant strains. Although there is evidence against the direct involvement of exonuclease V in the excision repair process (8), the enzyme might play a more important role in dimer excision when other exonucleases are missing. This fact should be borne in mind in the interpretation of the data presented here.

RESULTS

We were unable to construct the *recB21* derivative of strains containing the *polA546ex* mutation suggesting that this may be an inviable combination. Derivatives of these strains were therefore constructed containing the *recB270 recC271* temperature-sensitive mutations (5). As expected, these strains were found to be conditionally lethal. Strains containing only the *recB270 recC271 xseA7* mutations show slightly reduced survival at temperatures restrictive to derivatives containing *polA546ex*.

DNA degradation studies performed after low doses of UV reveal no significant differences between the wild-type and mutant strains after doses of 20 J/m^2. After higher UV doses (90 J/m^2) which caused 50-80% DNA degradation after 60 min of 43°C incubation in *polA546ex* and *polA546ex xseA7* strains, the *recB270 recC271* derivatives of these strains show only about 20% DNA degradation. DNA degradation in the *recB270 recC271 xseA7* strain was indistinguishable from wild-type at all UV doses. It is therefore possible to reduce postirradiation DNA degradation in these *polA546ex* derivatives by reducing the levels of exonuclease V.

UV survival studies show that the *recB270 recC271* derivatives of strains containing the *polA546ex* and *polA546ex xseA7* mutations are considerably more sensitive to UV irradiation than any of the strains from which they were constructed. The strain containing the mutations *recB270 recC271 xseA7* is only slightly more UV sensitive than strains containing the *recB270 recC271* mutation alone (data not shown).

Thymine dimer excision studies were performed on KLC124 (wild-type), KLC333 (*recB270 recC271 polA546ex*), and KLC341 (*recB270 recC271 polA546ex xseA7*). Cultures labeled with [^{3}H]-thymine were irradiated, warmed to 43°C, and samples were examined for thymine-containing dimers as a function of incubation time (Fig. 1). Our results thus far show that KLC333 (*recB270 recC271 polA546ex*) is slightly reduced in its ability to remove thymine dimers compared to the wild-type. This may mean that the apparent deficiency in dimer excision in the

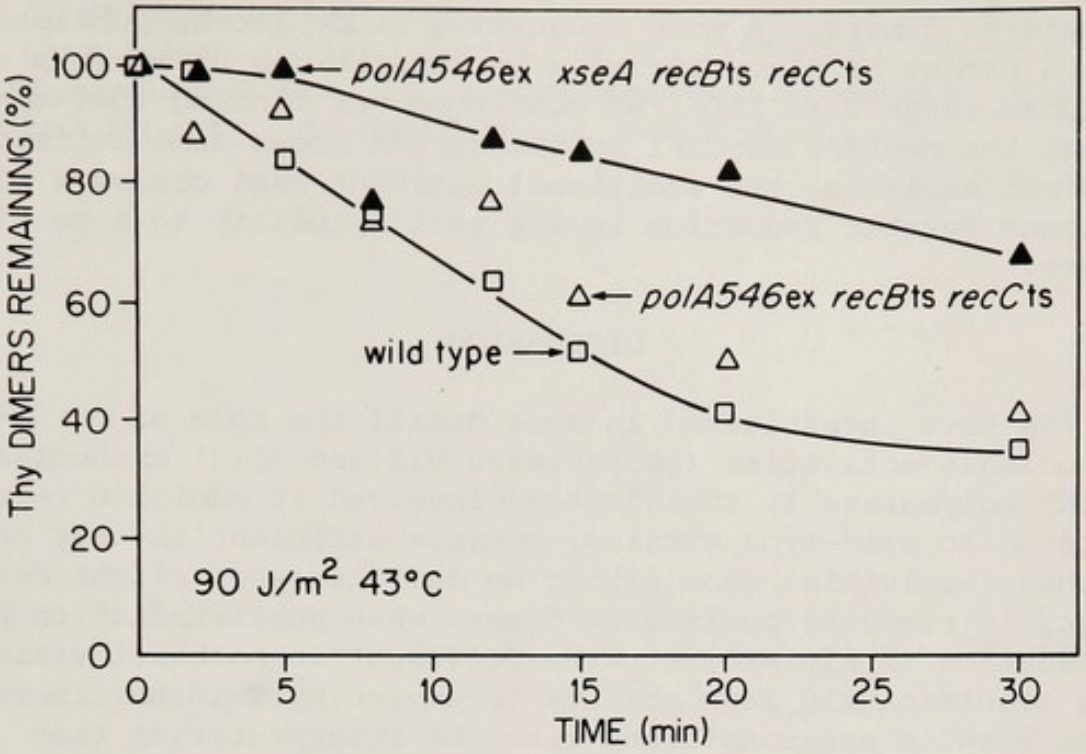

FIGURE 1. *Rate of Dimer Excision in Exonuclease Deficient Mutants.* Cultures of *E. coli* strains KLC124: *trpA33 rha thyA;* KLC333: *polA546ex recB270 recC271 trpA33 thyA;* and KLC341: *polA546ex recB270 recC271 xseA7 trpA33 thyA* were grown at 32°C to mid-log phase in M9 medium supplemented with required nutrients and ^{3}H-thymidine. The cells were chilled, harvested, and washed with M9 salts (without glucose or other additives) before irradiation with 90 J/m^2 UV light. The cells were warmed to 43°C for 5 min, nutrients were added (at t = 0) and samples were withdrawn at the indicated times. The cells were immediately chilled and ice cold NET buffer (0.1 M Tris HCl, pH 8, 0.01 M EDTA, 0.1 M NaCl) was added. The samples were collected by centrifugation, frozen, thawed and lysed by incubation with 400 µg/ml lysozyme for 30 min at 43°C and 0.2% sarkosyl for 10 min at 43°C. After 30 min of digestion with 250 µg/ml protease K at 50°C, the samples were chilled, TCA was added and the acid insoluble fraction digested with formic acid. Content of thymine dimers was determined by two-dimensional paper chromatography (9). At zero time 0.29% of the thymine was found as dimers. □ = KLC124; Δ = KLC333; ▲ KLC341.

polA546ex single mutant shown in our previous study (4) was largely due to postirradiation DNA degradation. Strain KLC341, however, is significantly deficient in dimer excision compared to wild-type and KLC333, suggesting that the additional deficiency in exonuclease VII in KLC341 is responsible

for a major reduction in the ability of this strain to excise pyrimidine dimers. A more exhaustive study (to be published) of the extent of dimer excision in this series of mutants over the dose range 0 to 160 J/m^2 confirmed our finding that although the *recB270 recC271 polAex* strain shows some deficiency in dimer excision, the additional mutation *xseA* causes a significant further reduction in the cell's ability to cope with dimers.

DISCUSSION

We have investigated in some detail the role of two 5'→3' exonuclease activities (exonuclease VII and 5'→3' exonuclease of DNA polymerase I) thought to be involved in excision repair. Compared to wild-type strains, mutants deficient in only one of these activities show either no deficiency or slight deficiency in removing pyrimidine dimers when postirradiation DNA degradation is minimal; strains deficient in both activities show significantly less ability to excise pyrimidine dimers. These results are consistent with the interpretation that both of these enzymes function in the excision process and are able to substitute for one another. Although excision is reduced in strains deficient in both exonuclease VII and the 5'→3' exonuclease of DNA polymerase I, it still occurs at a measurable rate. This may be due to residual enzyme levels of either or both of these activities or to other nucleases capable of dimer excision. The only other exonuclease activity of *E. coli* known to be capable of pyrimidine dimer excision *in vitro*, the 5'→3' exonuclease of DNA polymerase III, has not yet been evaluated *in vivo*. The possibility also exists that as yet unidentified nucleases participate in the excision step.

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CONDITIONS FOR CONSTITUTIVE AND INDUCIBLE GAP FILLING OF EXCISION AND POSTREPLICATION REPAIR IN TOLUENE TREATED *E. coli*

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ABSTRACT Toluene-treated *E. coli* were used to determine the conditions for the gap filling steps of excision and postreplication repair and to study the participation of inducible functions in these processes. Induction was performed *in vivo* by incubating cells under conditions that induce formation of SOS functions. Gap filling, when observed, was dependent on ATP and the presence of the four dNTP's.

INTRODUCTION

Alternate pathways for excision and postreplication repair have been postulated to stem from the existence of different mechanisms of the gap filling steps of these processes. Recent studies suggest that some of these mechanisms, e.g., gap filling by error prone postreplication repair and long patch excision repair, might require inducible functions (reviewed by Witkin [1]).

In the present study permeabilized *E. coli* were used to study the various mechanisms of gap filling and to determine their dependence on inducible functions.

RESULTS

Using toluene treated *E. coli* we have recently shown (2) that small patch gap filling of excision repair is saturated at low dNTP concentrations (2.2μM of each dNTP), results in incorporation of an average of 13-17 nucleotides per dimer excised, and is mediated by DNA polymerase I.

In toluene treated *polA* mutants patching of gaps takes place in coordination with incisions and is limited to repair of a small number of gaps (3, 4). Experiments performed as previously described (3, 5) show that repair of gaps in *polA* mutants increases with dNTP concentrations and is optimal at 33μM of each dNTP. The triphosphates, in addition to being

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incorporated also inhibit an ATP dependent DNA degradation, which takes place at incision sites (Miller and Ben-Ishai, unpublished). Since inhibition of DNA degradation by dNTP's is prevented when gap closure no longer occurs (>20 J/m^2), lack of control of DNA degradation by a SOS function may account for the low repair capacity of permeabilized polA mutants (1, 6).

To study the possible involvement of inducible SOS functions in large patch repair, intact polA cells were exposed to treatments that damage DNA or inhibit its synthesis. Following a period of incubation (30-90 min) in growth medium, cells were permeabilized, irradiated (7.5-30 J/m^2) and reincubated in a reaction mixture containing ATP, NAD and dNTP's. The gap filling process was analyzed by dNTP incorporation and accumulation of incision breaks. Inducing treatments included: (a) irradiation of intact cells with low fluences of UV light (1-3 J/m^2), (b) inhibition of DNA synthesis by novobiocin, and (c) incubation of a dnaBpolA mutant at the nonpermissive temperature. Of these treatments (a) and (b) were without any detectable effect; (c) resulted in i) inhibition of the repair synthesis which is observed without pretreatment and ii) an UV dependent dNTP incorporation that occurs in response to pretreatment and is dependent on protein synthesis (Table 1).

The gap filling step of postreplication repair was studied in toluene treated uvrA mutants in which DNA synthesis on a damaged template had occurred *in vivo* (see Fig. 1). Postreplication gap closure in toluene treated *E. coli* was found to be dependent on the presence of ATP, NAD and high concentrations of the four dNTP's. Under the conditions described in Fig. 2 pretreatment in the absence and presence of chloramphenicol leads to closure of 23 and 13 percent, respectively, of gaps initially present in newly synthesized DNA.

TABLE 1
UV STIMULATED dNTP INCORPORATION IN *E. coli* (dnaBpolA)[a]

pretreatment at 42°		UV (J/m^2)			
min	chloramphenicol	0	7.5	15	22.5
0	-	0.3	1.5	3.0	3.5
30	-	0.3	0.5	0.8	0.9
60	-	0.4	1.0	1.5	2.5
90	-	0.2	1.4	1.9	-
60	+	<0.2	<0.2	<0.2	0.3

[a] pmoles ^{3}H-dTTP incorporated at 42° in *E. coli* 1047 in the presence of ATP (1.3mM), NAD (0.5mM) and dNTP's (33μM each).

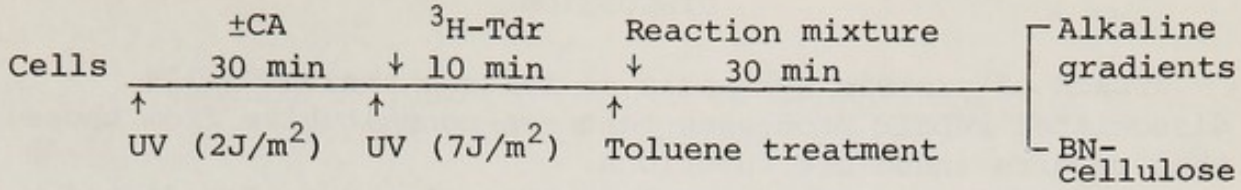

FIGURE 1. Protocol of experimental procedure: Log phase E. coli H514 (uvrA endoI) were exposed to UV ($2J/m^2$) and incubated in M-9 medium with or without chloramphenicol (CA). Following a second exposure to UV light, cells were pulsed with ^{3}H-Tdr for 10 min, resuspended in buffer, toluenized and incubated in a reaction mixture with or without ATP and dNTP's. Gap filling was determined by alkaline sucrose gradients and BN-cellulose chromatography.

Dependence of gap filling on ATP concentrations showed two optima, one at 0.4mM and the other at 1-1.6mM. In the presence of the low ATP concentration gap filling was almost twice that at the higher ATP concentrations (not shown).

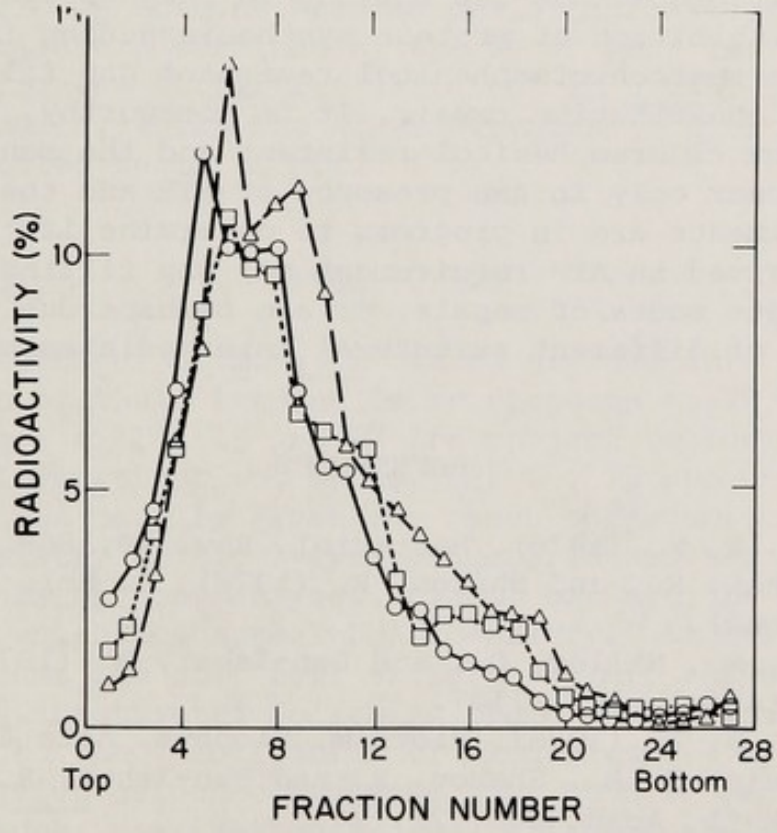

FIGURE 2. Alkaline gradient analysis of postreplication gap filling in E. coli (uvrA). Cells pretreated with (□——□) or without (Δ—Δ) chloramphenicol were incubated in a reaction mixture containing ATP (1.5mM); NAD (0.5mM) and dNTP's (33μM each), (o-o) denotes profile of newly synthesized DNA immediately after toluenization.

DISCUSSION

Lack of protein synthesis in toluene treated cells dissociates repair processes that are constitutive from those that require inducible functions.

Constitutive repair involves DNA polymerase I mediated small patch repair and an excision repair that occurs after low fluences in polA mutants and apparently does not involve filling in of gaps by long stretches of nucleotides (4).

To determine participation of inducible functions in long patch excision repair, polA strains were treated in vivo under conditions that induce SOS functions. It is perhaps of significance that of the various inducing treatments employed, only pretreatment of a dnaBpolA mutant showed any response. However, it still remains to be established if the UV stimulated dNTP incorporation observed after thermal pretreatment represents long patch repair.

About twenty percent of gaps which are formed on a damaged template are filled in toluene treated cells during incubation in the presence of ATP (1.6mM) and the four dNTP's (33μM each). Since this gap filling is only partially prevented by inhibition of protein synthesis during induction, it is feasible that chloramphenicol resistant gap filling represents constitutive repair. It is noteworthy, however, that both the chloramphenicol resistant and the sensitive gap filling, occur only in the presence of ATP and the four dNTP's.

Experiments are in progress to determine if the two optima observed in ATP requirement for gap filling, account for alternate modes of repair, or are perhaps due to generation of different structural intermediates of gapped DNA (7, 8).

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EFFECTS OF PHAGE INFECTION ON *Escherichia coli* EXCISION REPAIR MEASURED *in vitro*[1]

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ABSTRACT The effects of phage infection on the processes of excision repair in *E. coli* have been observed using cell free lysates and toluene treated cells. Infection of cells with lambda or T7 has little effect on the level of ATP-dependent UV specific endonuclease. Infection with T4*v* or T5 results in a rapid inactivation of UV specific activity. Infection with T3 results in the appearance of an apparently UV specific endonuclease which is ATP-independent. Measurement of UV induced DNA repair synthesis in toluene treated cells indicates that the UV endonuclease determined by the T4 v^+ gene is capable of replacing the activities deficient in *uvrA*$^-$, *uvrB*$^-$ and *uvrC*$^-$ cells, while the ATP-independent activity seen in T3 infected cells is not.

INTRODUCTION

Bacteriophages of *E. coli* can be divided into two classes on the basis of their intracellular response to UV irradiation. Bacteriophages λ, T1, T3 and T7 are subject to the processes of host cell reactivation, whilst T2, T4, T5 and T6 are not. The explanation usually given for these observations, that the unusual chemistry of the T-even phages renders the DNA unamenable to repair by host enzymes, cannot explain the behaviour of phage T5 which has normal DNA chemistry. It has been suggested (1) that the host cell excision repair enzymes may be specifically inactivated following phage infection, in a manner similar to the inactivation of exonuclease V by infecting T4 (2). *In vivo* evidence suggests that both T4 and T5 may be capable of such inactivation (3, 4), although the enzymes which are inactivated have not been identified. The enzymology of excision repair is quite well understood, the initial step being the introduction of a single strand break adjacent to a pyrimidine dimer by the ATP-dependent UV specific endo-

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nuclease specified by the uvrA$^+$, B$^+$ and C$^+$ genes (5, 6, 7). These studies exploit permeable cells and cell free lysates to follow the effects of phage infection on the activity of this endonuclease, and also investigate the interaction of phage-coded repair enzymes with those of the host.

Methods. Cells grown in K medium to approx. 2 x 10^8/ml were infected with phage at an m.o.i. of between 5 and 10. The procedures for toluene treatment of cells, irradiation and measurement of DNA repair synthesis have been described previously (8), as have the procedures for preparation of cell free lysates and subsequent assay of UV specific endonuclease activity (7). Standard reaction mixtures (130 µl) contained 5 nanograms of DNA, exposed to 200 J/m^2 of UV light, and were incubated for 15 minutes at 37°C in the presence of the enzyme. One unit of enzyme activity is defined as that which creates at least one single strand break in 63% of the DNA under these conditions. The amount of UV specific activity in each extract was corrected for the varying levels of non-specific endonuclease activity, assuming a Poisson distribution of breaks in the circle population and an equal degree of non-specific breakage in irradiated and unirradiated DNA.

Results. Table (1) shows the results of assaying ATP-dependent UV specific endonuclease in cell free lysates prepared from strain JC4583 uvr$^+$ endA at various times following addition of phage to the culture. Infection with T4v results in rapid inactivation of UV specific endonuclease, and after 15 minutes no activity can be detected. The presence of chloramphenicol during infection retards the inactivation, but does

TABLE 1. ATP-DEPENDENT UV SPECIFIC ENDONUCLEASE ACTIVITY IN LYSATES OF INFECTED CELLS OF JC4583 uvr$^+$ endA[a]

Condition of cells when lysate prepared	Infecting phage					
	T4v	T4v+cmp[b]	T5	T5+cmp	T7	λ
Uninfected			58.5	45.0	35.5	34.3
Infected 0 min.	63.8	28.8			37.6	29.8
5 min.					46.5	27.6
7.5 min.	3.4	14.6				
10 min.			8.8	43.5	33.1	34.7
15 min.	0	1.8				

[a] Figures given are standard units x100.
[b] Where indicated, 200 µg/ml chloramphenicol (cmp) was added to the cultures 20 minutes prior to infection.

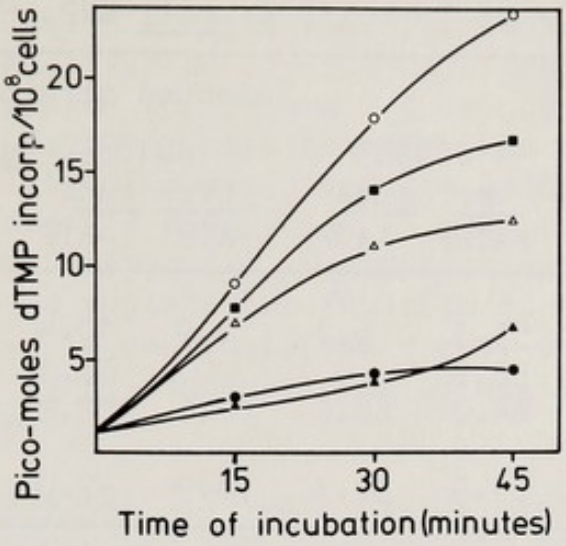

FIGURE 1. UV induced DNA repair synthesis in toluene treated cells of JC4583 uvr+ endA irradiated with 40 J/m^2 UV. Cells were infected 10 minutes prior to toluene treatment. All phage were inactivated with 400 J/m^2 UV prior to infection, to prevent phage replication. This level of irradiation does not affect inactivation of UV specific endonuclease measured in cell free lysates. ○ Uninfected cells, ● infected with T4v, △ infected with T7, ▲ infected with T5, ■ infected with T3.

not prevent it. Infection with T5 also causes endonuclease inactivation, which is prevented by chloramphenicol. Infection with either T7 or λ has no significant effect on the levels of enzyme activity.

To confirm these observations, the ability of infected cells to carry out extensive repair synthesis was investigated. Toluene treated cells of infected and uninfected JC4583 were irradiated and incubated in the presence of NMN to inhibit ligase. Under these conditions, extensive repair synthesis occurs, provided the initial single strand break is introduced by the uvr endonuclease (8). The results of such an experiment with uninfected cells, and cells infected with T3, T4v, T5 and T7 are shown in Figure 1. Infection with either T4v or T5 inhibits repair replication, while infection with T7 or T3 does not.

The effect of T3 infection on UV endonuclease measured in lysates is given in Table 2. An apparently UV specific, ATP-independent nuclease appears in T3 infected cells provided chloramphenicol is not present. Figure 2 shows that the v gene endonuclease of T4 can substitute for the uvrABC endonuclease of E. coli in creating the initial single strand break while the activity detected in T3 infected cells cannot.

TABLE 2. UV SPECIFIC ENDONUCLEASE ACTIVITY[a] IN LYSATES OF T3 INFECTED E. coli K12.

Condition of cells when lysate prepared	Infected strain JC4583 uvr+ endA +ATP	JC4583 uvr+ endA -ATP	AB1885 uvrB end+ +ATP	AB1885 uvrB end+ -ATP	JC4583+cmp[b] +ATP	JC4583+cmp[b] -ATP
Uninfected	53.3	6.1	4.9	10.4	53.4	14.0
Infected 0 min.	52.9	0	0	10.1		
7.5 min.	64.0	68.0	82.6	52.2		
10 min.					46.8	14.6
15 min.	64.9	57.3	78.9	47.0		

[a]Figures given are standard units x100.
[b]Chloramphenical (cmp) was added to 200 µg/ml 20 minutes prior to infection.

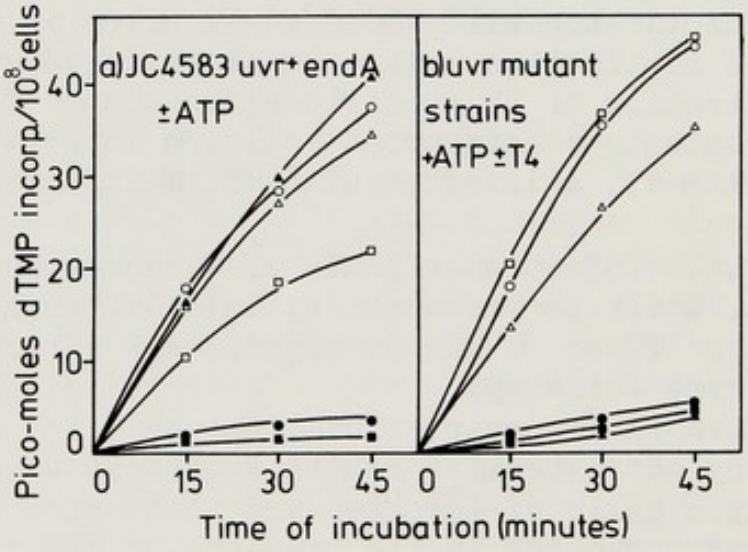

FIGURE 2. DNA repair synthesis in irradiated toluene treated cells, and the effects of phage infection. Cells were infected 10 minutes prior to toluene treatment, and irradiated with 40 J/m^2 UV. In the case of T4, all infections were carried out in the presence of 200 µg/ml chloramphenicol. Symbols are as follows.

Section (a)
- ○ Uninfected cells +ATP
- ● Uninfected cells -ATP
- △ T4v+ infected +ATP
- ▲ T4v+ infected -ATP
- □ T3 infected +ATP
- ■ T3 infected -ATP

Section (b)
- ○ AB1886 uvrA, infected with T4v+
- ● AB1886 uvrA, uninfected
- △ AB1885 uvrB, infected with T4v+
- ▲ AB1885 uvrB, uninfected
- □ AB1884 uvrC, infected with T4v+
- ■ AB1884 uvrC, uninfected

Discussion. Infection of E. coli with bacteriophages T4v and T5 results in a rapid inactivation of the uvr endonuclease. This inactivation depends upon protein synthesis. Infection with phages T3, T7 and λ does not result in inactivation of this enzyme. These observations correlate well with the in vivo ability of these phages to undergo host cell reactivation. Infection with phage T3 results in the appearance of an apparently UV specific ATP-independent endonuclease, which is unable to replace the function of the host enzyme in excision repair. In contrast, the v gene endonuclease of T4 can replace the uvrABC endonuclease in cells mutated in any of these three genes.

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REPAIR OF DNA DAMAGE IN MYCOPLASMAS [1]

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ABSTRACT Studies of the repair of UV induced DNA damage in mycoplasmas have examined excision repair, photoreactivation, and repair of photodynamic inactivation. *A. laidlawii* cells were found to have all of these repair capabilities. These cells can also host cell and UV reactivate irradiated mycoplasmaviruses. *M. gallisepticum* cells lack both excision repair and photoreactivation mechanisms. Other *Mycoplasma* species may also lack repair mechanisms.

INTRODUCTION

The mycoplasmas are the smallest free-living cells. Their repair capabilities have been studied to examine the ubiquity of DNA repair processes in cells with such small genomes. These prokaryotes do not have cell walls: each cell is bounded by a single lipoprotein plasma membrane (e.g., ref. 1). The various mycoplasma isolates have been classified into six genera. Of these, the molecular mechanisms for the repair of ultraviolet (UV) light induced DNA damage have been studied in only two species, *Acholeplasma laidlawii* and *Mycoplasma gallisepticum*. *A. laidlawii* chromosomal DNA has a molecular weight of 1.0×10^9 and is 33% GC, while *M. gallisepticum* DNA is 0.46×10^9 daltons and 35% GC (reviewed in ref. 1). *A. laidlawii* is the indicator host for both single and double stranded DNA mycoplasmaviruses (e.g., ref. 2).

EXCISION REPAIR

A. laidlawii. Studies of liquid holding recovery of UV irradiated *A. laidlawii*, held in the dark in buffer, have shown that these cells are capable of dark repair. During holding the cell survival fraction increased with time and

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reached a maximum level after 45 min of incubation. The extent of recovery was dependent on the UV dose used to inactivate the cells (3,4,5). Chloramphenicol (80-90 µg/ml) added to the cells either before or after UV irradiation reduced liquid holding recovery to about 10% of that of untreated cells (3). However, when chloramphenicol was added before irradiation dark repair was less efficient than when it was added after irradiation. Nalidixic acid (100 µg/ml) added to the cells before irradiation reduced liquid holding recovery to about 10% of that of untreated cells; but, when added after irradiation to cells in holding buffer, only about 50% reduction in recovery was observed (3). Acriflavine (0.5 µg/ml) completely eliminated liquid holding recovery (5).

Studies of the DNA from UV irradiated *A. laidlawii* have shown excision repair during liquid holding recovery. Smith and Hanawalt (6) grew UV irradiated cells in medium containing bromodeoxyuridine and analyzed the DNA by sedimentation to equilibrium in cesium chloride gradients. These experiments demonstrated repair replication, involving non-conservative synthesis of small single strand regions of the DNA. The average size of a repaired region was estimated to be 150-600 nucleotides. Das *et al.* (3) used velocity sedimentation in alkaline sucrose gradients, to denature the cell DNA and measure the number average molecular weight of single stranded DNA from UV irradiated cells during liquid holding recovery. The molecular weight decreased during the first 20 min of holding and then began to increase. This indicates that during liquid holding recovery single strand breaks are made in the DNA and then repaired. Similar alkaline sucrose gradient studies of acriflavine treated irradiated cells during liquid holding have shown that in these cells single strand DNA breaks are made but not repaired (5). All of these data indicate that *A. laidlawii* cells have an excision repair system.

M. gallisepticum. The UV survival curve of these cells is almost exponential with a zero-dose intercept about 1.5, close to the value of 1.4 which would be expected for exponentially growing cells each containing a single genome copy (4). The absence of a shoulder on the survival curve suggested that these cells might not be able to repair UV induced DNA damage. Therefore, experiments were carried out to investigate dark repair in these cells.

No recovery could be found during liquid holding of UV irradiated *M. gallisepticum* (4). Analysis of the DNA from irradiated cells by velocity sedimentation in alkaline sucrose gradients showed that there was no change in the molecular weight of the single stranded DNA during liquid holding recovery. However, *Micrococcus luteus* UV-endonuclease treatment

(which had no effect on the DNA from unirradiated *M. gallisepticum*) caused a reduction in the molecular weight of single stranded DNA from UV irradiated *M. gallisepticum*, with no measurable loss of acid precipitable material(4). Therefore, UV irradiated *M. gallisepticum* DNA must contain pyrimidine dimers which the cell is unable to excise and repair. These cells must lack a UV-endonuclease specific for pyrimidine dimers.

Mycoplasmaviruses. *A. laidlawii* has been shown to be capable of both host cell (HRC) and ultraviolet (UVR) reactivation of UV irradiated mycoplasmaviruses (7). HCR was examined by comparing the survival of a UV irradiated double stranded DNA mycoplasmavirus plated on both untreated and acriflavine treated cells. Decreased virus survival on the acriflavine treated cells demonstrated HCR. Consistent with HCR being an excision repair process, no reactivation was observed in studies of a UV irradiated single stranded DNA mycoplasmavirus.

UVR was studied by comparing the survival of UV irradiated virus plated on untreated cells and on cells that had received a small UV dose before plating. The UV irradiated cells gave increased survival of both single and double stranded DNA mycoplasmaviruses, showing UVR. In bacteria, UVR involves a DNA repair mechanism different from either excision or recombinational repair (8,9) and may be due to an inducible error-prone repair system (10). Therefore, the finding of UVR by *A. laidlawii* suggests that such an inducible repair system may be present in these cells.

Other mycoplasmas. Although no repair studies have been reported for other mycoplasmas, various data suggest that *Mycoplasma* species might lack dark repair. The principal DNA polymerase activity has been isolated from *M. orale* and *M. hyorhinis* and found to lack exo- and endonuclease activities and to be unable to excise thymine dimers (11). The UV inactivation curves of eight *Mycoplasma* species (on agar plates) were all found to be almost exponential (12,13).

PHOTOREACTIVATION

Photoreactivation of UV induced DNA damage has been demonstrated in several *A. laidlawii* strains (3,4,6,14). UV irradiated cells exposed to visible light show greater survival than irradiated cells not exposed to visible light. In a study of the possible cumulative effects of liquid holding recovery and photoreactivation of *A. laidlawii*, it was found that both repair mechanisms could only increase cell survival to a given saturation level (3): this level is dependent on

the inactivating UV dose. The result indicates that both dark repair and photoreactivation act on the same lesion.

Similar studies on *M. gallisepticum* showed no photoreactivation (4). Instead, both unirradiated and UV irradiated cells were found to be inactivated by the visible (fluorescent) light used in these experiments. The slopes of these photoinactivation curves were the same, for both unirradiated cells and cells irradiated with different UV doses. Therefore, photoinactivation is not due to a synergistic action between the effects of UV and visible light.

PHOTODYNAMIC INACTIVATION

Exposure of toluidine blue (15) or acriflavine (16) treated *A. laidlawii* cells to visible light leads to photodynamic inactivation (PDI). PDI was also observed for acriflavine treated *M. gallisepticum* (16).

In the studies of *A. laidlawii* using acriflavine, it was found that some of the damage induced by PDI could be repaired if the cells were incubated in the dark (16). The extent of recovery was dependent on the amount of PDI; no recovery was observed when PDI was sufficient to give 90% or greater cell inactivation. Analysis of the DNA of cells after PDI showed strand breaks which could be repaired by liquid holding.

CONCLUSIONS

In addition to the repair studies reviewed here, two other types of effects of UV irradiation on *A. laidlawii* cells have been reported. First, an *A. laidlawii* variant with a REP^- phenotype (no longer able to propagate single stranded DNA mycoplasmaviruses, but retaining the ability to grow double stranded DNA mycoplasmaviruses) was found to have an increased sensitivity to inactivation by UV relative to wild-type cells (17). Second, *A. laidlawii* virus indicator strains can host modify and restrict double stranded DNA mycoplasmaviruses. However, if an unmodified virus is plated on a restricting host, which has previously been mildly UV irradiated (survival greater than 70%), an increased number of plaques is seen, relative to plating on unirradiated cells (18). It is not known whether this UV effect is due to increased modification, decreased restriction, or both in irradiated cells.

In summary, it appears that *A. laidlawii* cells have most if not all of the capabilities, for the repair of UV induced DNA damage, as the eubacteria. However, it has not been possible to demonstrate any repair mechanisms in *M. gallisepticum*. Other *Mycoplasma* species may also lack repair mechanisms.

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ROLE OF THE GENE *mtcA* IN THE RESISTANCE OF *MICROCOCCUS RADIODURANS* TO THE LETHAL EFFECTS OF MITOMYCIN C AND ALKYLATION MUTAGENESIS[1]

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ABSTRACT A strain, 302, of *Micrococcus radiodurans* mutant in *mtcA* is very sensitive to the lethal effects of mitomycin C (MTC) and decarbamoyl MTC but is fully UV resistant. Alkaline sucrose gradient analysis of DNA from bacteria treated with 7-bromomethylbenz [*a*]-anthracene (BrMBA) indicates that 302 is unable to initiate excision repair of BrMBA-DNA adducts. 302 is hypermutable by methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). Both the wild type and 302 excise 7-methylguanine (7-MeG) and 3-methyladenine (3-MeA) from MNNG-methylated DNA but only the wild type excises O^6-methylguanine (O^6-MeG). The *mtcA* gene product is therefore involved in the initial recognition and/or incision events in excision repair of MTC-mono- and di-adducts and also certain methyl and ethyl products in DNA.

INTRODUCTION

M. radiodurans is resistant to both the lethal and mutagenic effects of many DNA-damaging agents (1). One mechanism of mutagenesis is that caused by the replication of chemically altered bases such that mispairing occurs (2). Certain alkylation products are in some systems enzymatically excised and unless removed from DNA may be a source of infidelity. A mutant of *M. radiodurans* has been isolated, sensitive to the lethal action of MTC, which is hypermutable by MMS, EMS and MNNG. We report here some of its properties.

RESULTS

Compared to *M. radiodurans* wild type, 302 was very sensitive to the lethal effects of MTC and decarbamoyl MTC, slightly sensitive to BrBA, *N*-acetoxy-*N*-acetyl-2-aminofluorene (AAAF),

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TABLE 1
SENSITIVITY OF *M. RADIODURANS* WILD TYPE AND 302 TO KILLING BY SOME DNA-DAMAGING AGENTS.

Agent	D_{37} dose Wild type	302
MTC (mM.h)	12.7	0.32
Decarbamoyl MTC (mM.h)	532	10.9
MMS (mM.h)	58	64
EMS (mM.h)	726	340
MNNG (μM.h)	1061	177
BrMBA (nM.min)	562	187
AAAF (μM.min)	63	22
Hydroxylamine (mM.h)	810[a]	240
Nitrous acid (mM.h)	3.4[a]	1.5
γ-rays (krad)	700	670
UV (Jm^{-2})	1200	1190

[a]Data from Ref. 1.

hydroxylamine, nitrous acid, EMS, MNNG and γ-rays but had the same resistance to UV and MMS (Table 1). In constrast to the wild type, which was mutated only to a slight extent by MMS and EMS, 302 was very sensitive to the mutagenic effects of both agents (Fig. 1). Although the wild type was readily mutated by MNNG, 302 was even more sensitive by a factor of about 50 (Fig. 1). 302 was also slightly hypermutable by hydroxylamine but was no more mutable than the wild type to any other agent examined.

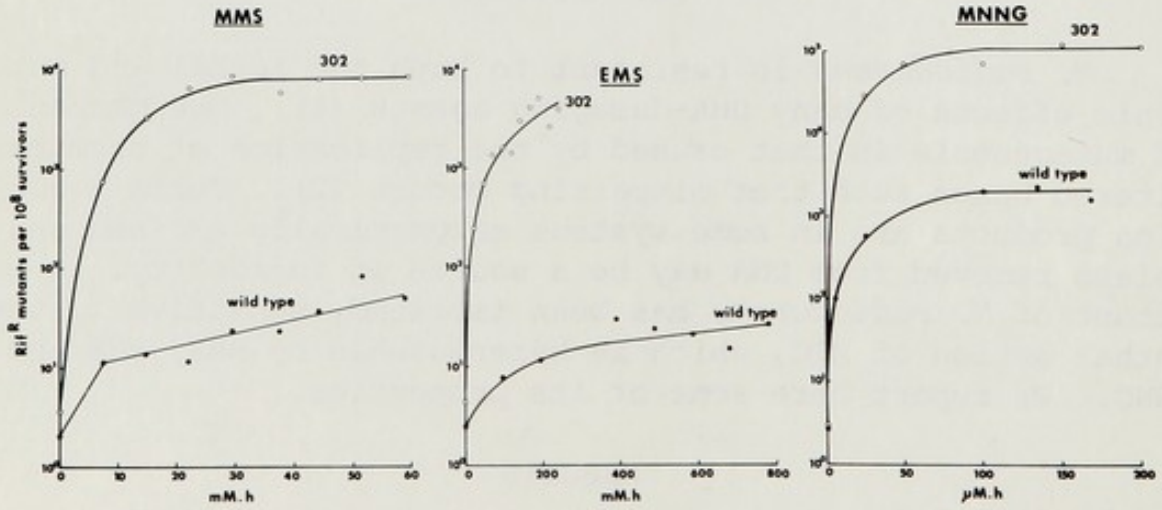

FIGURE 1. The ability of MMS, EMS and MNNG to induce rifampicin-resistant mutants of *M. radiodurans* wild type and 302.

Alkaline sucrose gradients of the DNA from bacteria treated with BrMBA show that in the wild type the DNA has been enzymatically attacked to produce single-strand breaks or alkali-labile sites. DNA from BrMBA-treated 302 sediments close to control positions (Fig. 2) probably due to a deficiency in a correctional endonuclease or *N*-glycosidase activity.

When 302 was treated with [*methyl*-^{3}H]-MNNG and methylated purines identified by paper chromatography (4) 75% of DNA methylations were at N-7 of guanine, 8% at O-6 of guanine and 2% at N-3 of adenine. On incubating the bacteria for 3 h in growth medium following methylation the amount of 7-MeG was reduced by 83% and the amount of 3-MeA reduced to an undetectable level. In contrast, the amount of O^6-MeG remained constant. After incubation of MNNG-treated wild type 7-MeG was found in approximately the same amount as in 302. 3-MeA and O^6-MeG, however, were not detected.

DISCUSSION

The role of the *mtcA* gene product appears to involve initial recognition and/or incision events in the excision repair of MTC mono- and di-adducts and promutagenic lesions produced by methylating and ethylating agents. The rates at which the methylated purines 7-MeG and 3-MeA are removed from the DNA of *M. radiodurans* greatly exceeds the rates of spontaneous hydrolysis of the *N*-glycosidic bond (5) and implies

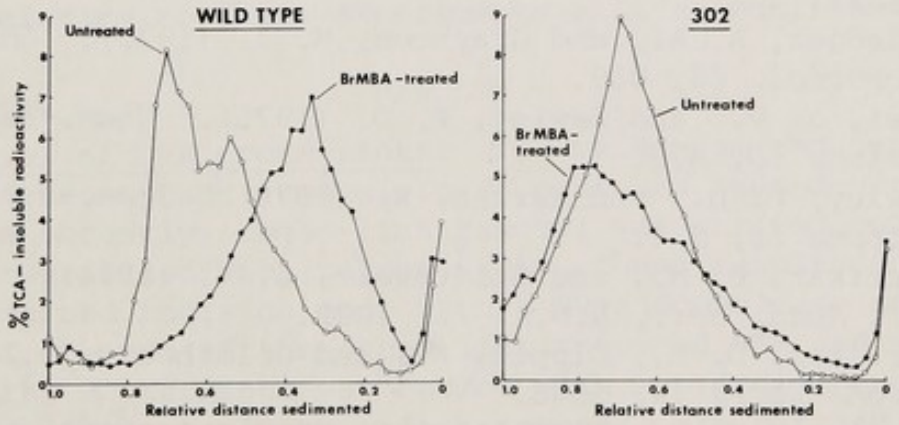

FIGURE 2. Alkaline sucrose gradients of DNA from *M. radiodurans* wild type and 302 treated with BrMBA. Bacteria, labelled with ^{3}H-thymidine, were treated with 2.5 μg ml^{-1} BrMBA for 20 min at 30°, spheroplasts prepared (3) and lysed on top of 4.5 ml 5-20% (w/v) sucrose gradients containing 0.1 M NaOH and 0.1 M NaCl. Sedimentation was for 105 min at 30000 r.p.m., 20°, in an SW 6x5.5 ml rotor of an M.S.E. Superspeed 65 ultracentrifuge.

that in the wild type and 302 7-MeG and 3-MeA are excised enzymatically. O^6-MeG, which is chemically stable in DNA, is enzymatically excised in the wild type but not, however, in 302.

The specificity of the *mtcA* gene product resembles endonuclease II from *Escherichia coli* in its recognition of O^6-MeG, methylbenz [*a*] anthracenyl residues and some γ-ray damage in DNA (6-8). The defective removal of O^6-MeG seen in 302 has also been reported in target tissue for neoplastic transformation (9) and in xeroderma pigmentosum cells (10). The promutagenic status of O^6-MeG has been demonstrated *in vitro* (11) but whether its persistance in 302 accounts for the hypermutability has not yet been ascertained since the pattern of removal of other alkylation products is not known. However, it may be concluded that neither 7-MeG nor 3-MeA are contributing to the hypermutability shown by 302.

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ON THE MECHANISM FOR REPAIR OF CROSS-LINKED DNA IN *E. coli* TREATED WITH PSORALEN AND LIGHT[1]

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ABSTRACT This article describes our recent progress in studies on the mechanism of repair of cross-linked DNA in *E. coli* treated with psoralen and light. Some of the reactions involved in this complex repair sequence were carried out in permeabilized cells and *in vitro* with purified enzymes. Other studies in whole cells indicate that genetic recombination is involved in this sequence, and that successive repair events controlled by *recA* occur singly in time, in clusters that may correspond to domains of the folded chromosome.

INTRODUCTION

Psoralens bind photochemically to DNA forming adducts to single strands and interstrand cross-links. Cross-linking damages are repaired by a complex mechanism which appears to involve cross-link removal followed by a recombinational event to restore the intact base sequence (1). Outlined below are results of some studies on repair and recombination in *E. coli* treated with psoralen and light. The details and procedures relating to these experiments will be published separately.

RESULTS

Removal of DNA cross-links. The mechanism and genetic control of cross-link removal in *E. coli* has been described (2). More recently, cross-link removal was carried out in cells permeabilized with toluene (3). These results indicated that the strand incision made by the UV-endonuclease on the 5' side of one arm of the cross-link required ATP and Mg^{++}, and that the 3' terminus was formed with a hydroxyl group. DNA strand cutting and cross-link removal were carried out *in vitro* by tandem reactions with endonuclease V(4) of phage T4 infected *E. coli* or the UV endonuclease of *Micrococcus luteus* (5) and DNA polymerase I of *E. coli*. These reactions occurred with both supercoiled and linear DNA of Col El plasmid DNA, and strand cutting by the *M. luteus* enzyme was not detected in

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supercoiled Col El containing an average of more than 40 monoadducts and less than one cross-link. These results and the studies with whole and permeabilized cells suggested that strand cutting at monoadducts was not appreciable. After the first strand cutting reaction by a repair endonuclease, the cross-link can be removed by the 5'-3' exonuclease activity of DNA polymerase I of E. coli. This second reaction did not occur in whole or permeabilized cells with mutations at uvrD, but the reaction occurred with normal efficiency using highly purified DNA polymerase I acting on cross-linked DNA previously cut by one of the above repair endonucleases.

After removal, a gap of about 1000 nucleotides is opened near each cross-link. These gaps appeared to be greatly reduced in size in recF⁻ strains. This was concluded from experiments on the susceptibility of DNA at various stages of repair to digestion by S1 nuclease of Asperigillus oryzae. Products were acid-soluble nucleotides and double strand fragments of length equal to the average cross-link spacing. These reactions were also attempted on nucleoids isolated at different stages of repair. Surprisingly, it was found that folded chromosomes retained their fast sedimenting behavior even after cross-link removal. Such nucleoids (isolated in 0.1 N NaCl) were resistant to treatment with S1 nuclease, but could be cut to segments as described above if the isolated nucleoid preparation was previously treated with 2 N NaCl or proteinase K. One possibility is that proteins were bound to DNA in the gap produced after cross-link removal. The sequence of events for cross-link removal and the structure of the DNA product suggested by these results are shown in Figure 1.

Completion of repair by recombination. After cross-link removal, the DNA can be denatured by alkali into single strand segments having an average length about equal to twice the cross-link spacing (1,2). On further incubation in cells, cut strands were joined into high molecular weight DNA, and this reaction is mediated by most of the gene products involved in genetic recombination. This strand rejoining reaction appears to provide a convenient assay for completion of recombination, and does not require formation of viable progeny. Rifampacin and novobiocin blocked further strand rejoining when added any time during repair, but additions of chloramphenicol had little or no effect.

A notable feature of the sedimentation profile of DNA released from cells at all stages of repair was a bimodal distribution of sizes. This appeared to be composed of DNA strands fully cut after cross-link removal and high molecular weight strands similar to those from control cells. Such behavior would be expected if successive damages were repaired progressively around the chromosme, or as clusters which

consist of 2-4% of the genome. The rate of strand rejoining per genome appeared to be constant at about 0.4 and 0.9 min^{-1} at 32 C and 37 C, and was not dependent on the number of cross-links present.

The kinetics for completion of the step requiring $RecA^+$ were determined using E. coli recA200 ($RecA^+$ at 32 C, $RecA^-$ at 42 C) shifted between permissive and restrictive conditions at various times during repair. Increases in survival taken as a measure of completion the $RecA^+$-dependent step before the temperature shift suggested that repair was occurring at a constant rate independent of the number of cross-links present on each genome. The rate constant was about 0.4 min^{-1} and initial increases in survival occurred within a few mins after the cross-linking treatment. These results are consistent with $RecA^+$-dependent repair reactions on each genome occurring singly for each successive damage encountered (9).

The rate for completion of the $RecA^+$-dependent reaction and the strand rejoining reaction appeared to proceed at the same rate. Since completion of repair of each damage (as judged by strand rejoining) likely involves a series of biochemical reactions, it appears that the rate-determining step occurs at the reaction controlled by $RecA^+$ or at an earlier step.

Theoretical calculations based on the recombinational repair mechanism described below were made in order to relate the average number of damages per genome, kinetics for completion of repair resulting in survival, the extent of DNA strand rejoining, the survival curve, and the molecular weight distribution of DNA at various stages of repair. An excellent correlation between experimentally-determined and calculated values was found for the following model (9). Recombination events occur singly on each chromosome. Successive events occur in a progressive fashion around the genome, or within regions consisting of 2-4% of the genome (such as supercoiled domains of the folded chromosome). Each repair event requires a constant length of time not dependent on the number of damages present. Unrepaired genomes decay to forms not rescuable by recombination in a reaction with rate proportional to the number of sites of unrepaired damage. Successive recombination repair events occurring in a clustered fashion within supercoiled domains of folded chromosomes could provide a reasonable mechanism for synapsis, as suggested by Holloman & Radding (7) and Meselson & Radding (8). Reactions occurring singly could serve to reduce errors and possible tangles among the products. A mechanism of how strand exchanges during the repair of cross-linked DNA might occur is shown in Fig. 2. At the top are shown homologous loops of two folded chromosomes. The left loop carries a cross-linking damage. At the first step, the cross-link is removed as depicted in Fig. 1. The

stable ends so produced invade the supercoiled domain of the intact homolog, as shown. Ensuing strand exchanges and repair synthesis restore the intact base sequence to the damaged region. After removal of the second arm of the cross-linking damage, repair can be completed, and the loops in each domain are again supercoiled.

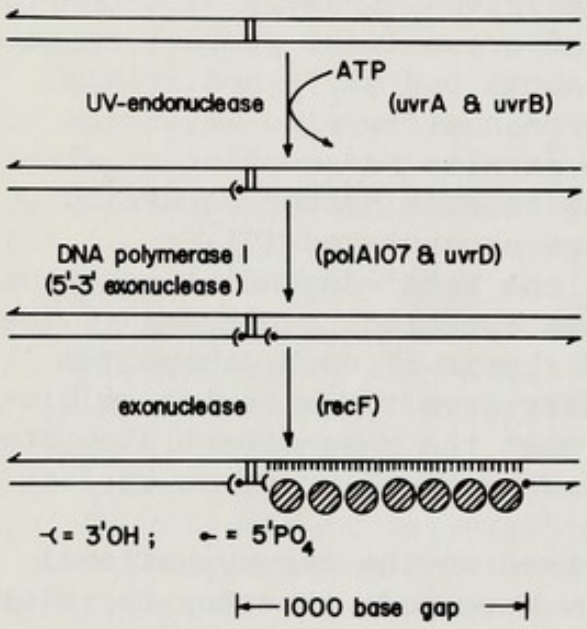

Figure 1.

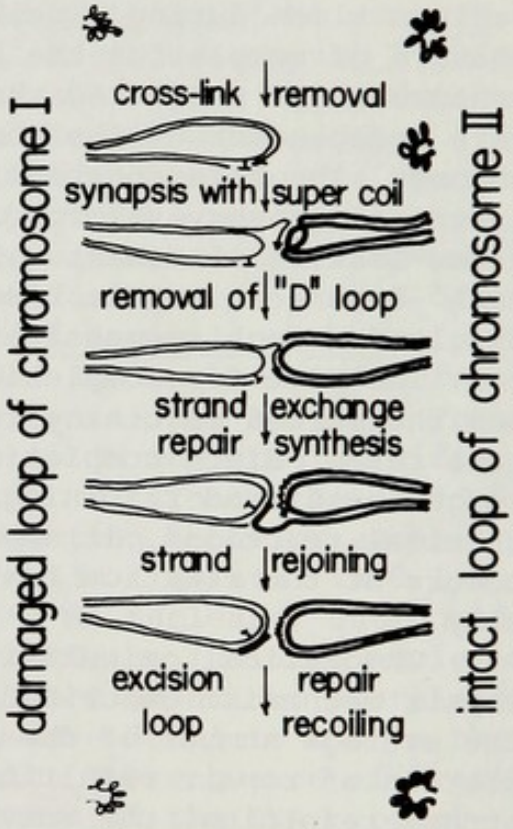

Figure 2.

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ENDONUCLEASE FROM CALF THYMUS THAT ACTS ON DNA IRRADIATED WITH LOW UV DOSES[1]

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ABSTRACT We describe the isolation of an endonuclease that nicks phage PM2 DNA irradiated with UV doses producing one to two pyrimidine dimers per molecule. The enzyme is Mg^{+2} dependent, stimulated by low concentrations of NaCl (5-15 mM), does not bind to DEAE Sephadex and copurifies with a nonspecific endonuclease. The enzyme can be recovered from thymus glands kept frozen at -20°C for at least two weeks but its activity in column fractions is lost within three days after the homogenization of the tissue.

INTRODUCTION

Several types of mammalian cells efficiently remove pyrimidine dimers from their UV-irradiated DNA. This dimer excision process is stated by an endonucleolytic incision in the vicinity of the dimer. Dimer-specific endonucleases have been extensively purified from phage T4 infected cells and some bacterial cells (for review see 1). However, attempts to isolate a dimer-specific endonuclease from mammalian cells have been unsuccessful. The UV-dependent endonucleolytic activities purified from calf thymus (2), rat liver (3) and human lymphoblasts (4,5) recognize photoproducts other than dimers. They act on UV- and x-irradiated DNA and enzymatic monomerization of the dimers does not affect the substrate properties in nucleolytic assays (2). The endonucleases were assayed on DNA molecules exposed to high UV-doses introducing $\geq$ 30 dimers per molecule. Such molecules contain some alkali stable photoproducts other than dimers that are recognized by these nucleases.

Bovine cells have an efficient dimer excision system (6). Fibroblasts not only remove a large fraction of dimers

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but also rapidly introduce large numbers of strands breaks which disappear during later steps of the repair process. Hence we tried to isolate a dimer-specific endonuclease from calf thymus. To maximize the possibility that pyrimidine dimers are the photoproduct recognized by the enzyme, phage PM2 DNA used as a substrate for assay was exposed to UV doses less than 15 J/m^2. Such a DNA contains not more than 2-3 dimers per molecule and seems to be free from the other types of photoproducts that appear after much higher doses of UV (7). Hence we believe that the activity we measure in UV-irradiated DNA is a dimer specific one.

METHODS AND RESULTS

Fresh calf thymus removed from the animal immediately after slaughter, was cooled to 0°C for immediate use or kept frozen at -20°C for up to 3 weeks. 80 g of the thymus, cut into small pieces, was homogenized for 15 sec in a Sorvall Omni-mixer in 60 ml of 50 mM Tris-HCl, pH 8.0-10% ethylenglycol-5 mM -mercaptoethanol. Sediments were discarded after 10 min centrifugation at 12000g and 30 min at 140000g. The clear supernatant (crude extract) was used for enzyme testing and further isolation procedures.

Endonucleolytic activity was assayed by the conversion of phage PM2 DNA to nicked circles. The 100 µl assay mixture routinely contained 10 mM Tris-HCl, pH 8.0-10 mM NaCl- 10 mM $MgCl_2$ and about 0.1 µg ^3H-DNA (4000 cpm). After 5-10 min incubation at 37°C with enzyme solution, the amount of nicked circles was determined by a filtration assay or by velocity centrifugation in alkaline sucrose gradients. All enzyme samples were tested in parallel on UV-irradiated and unirradiated DNA.

Under our assay conditions crude extracts of calf thymus reveal two endonucleolytic activities: a non-specific and a UV-specific one. In crude extracts the second is only a very small addition to a very high endonucleolytic activity which nicks unirradiated DNA. Both enzymes do not bind to DEAE Sephadex and appear in the first flow-through fractions of the column. They are associated with the first peak of proteins and continue to elute until the second peak of proteins comes off the column. Application of a linear salt gradient (0-0.5 M NaCl) releases no further endonucleolytic activity observed under our assay conditions.

Further purification was achieved by rechromatography of the active fractions on a second DEAE Sephadex column. Both enzymes again appear in the flow-through fractions of the column together with a small peak of proteins. The UV-specific activity is high against a relatively low

background of nonspecific activity. Both activities can be further separated by affinity chromatography on DNA Sepharose. The UV-specific activity elutes first whereas the non-specific activity comes off at a slightly higher KCl concentration (25 mM).

The UV-specific activity is Mg^{2+} dependent and 5-15 mM MgCl is optimal. It is inactive in the presence of 10 mM EDTA. Small concentrations of NaCl (5-15 mM) stimulate its activity. Increasing the UV-dose to PM2 DNA increases the number of nicks made by the calf thymus enzyme and the numbers of nicks made by the enzyme are close to those made by the M. luteus UV-endonuclease (Fig. 1).

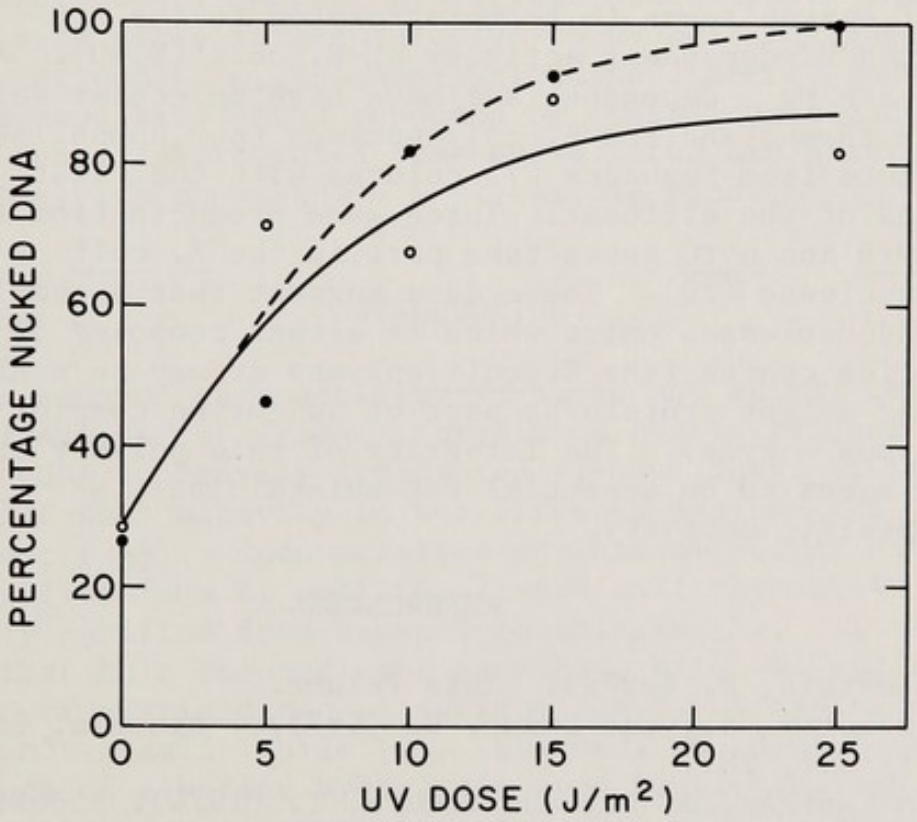

FIGURE 1. The percentage of nicked PM2 DNA as a function of dose for the UV specific activity of calf thymus (--0--) and for the M. luteus UV-endonuclease (--•--).

The UV-specific endonuclease can be recovered from thymuses kept frozen at -20°C for several weeks. The bovine enzyme seems to be more freeze resistant than its human analogue which looses its activity completely after freezing the cells (8). The calf thymus enzyme becomes labile during purification and is rapidly lost from column fractions kept

at ice temperature. All enzyme fractions were kept at ice temperature and contained 20% of glycerol which considerably enhanced the stability of the enzyme.

DISCUSSION

The UV-specific activity of calf thymus seems to recognize pyrimidine dimers since the number of nicks it makes is comparable to the number of dimers and to the nicks made by the dimer recognizing M. luteus enzyme. The calf thymus enzyme seems different from the dimer-specific endonucleases isolated from M. luteus and phage T4 infected cells which are proteins of low molecular weight (12000-16000) active in the presence of EDTA (see review 1). The calf enzyme seems to be more related to the ATP stimulated UV-dependent activity of E. coli (9,10). Both enzymes are Mg^{2+} dependent and have high molecular weight. The calf thymus and the E. coli enzymes (our unpublished data) elute from Sephadex G75 columns with the first fractions of the effluent. Three gene products from the uvrA, uvrB and uvrC genes take part in the E. coli UV-endonuclease (10). These data suggest that a complex type of UV-endonucleases exist which is either composed of several polypeptide chains (the E. coli enzyme) or may be a high molecular weight protein or part of an enzyme complex (the calf thymus enzyme). The integrity of this polymer or complex seems to be essential for maintaining its dimer-specific activity.

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ON THE NATURE OF THE HUMAN ENDONUCLEASE ACTIVITY DIRECTED AGAINST ULTRA-VIOLET IRRADIATED DNA[1]

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ABSTRACT Alkali-labile sites in UV irradiated DNA were detected by prolonged alkaline hydrolysis but were not detectable by shorter hydrolysis procedures. The number of such sites formed relative to UV dose corresponded to the number of the non-pyrimidine dimer UV endonuclease sensitive sites reported by other investigators. Evidence is presented that these sites are altered bases with an unstable N-glycosidic bond.

INTRODUCTION

The endonuclease activity in human and animal cells directed against UV (254nm) irradiated DNA is evident only when the DNA is heavily irradiated (1-4). Thus, it is probable that this activity is not directed against the pyrimidine dimer (3-5). Endonucleases against non-dimer sites have been purified from E. coli (6, 7) and calf thymus (4) and partially purified from human lymphoblasts (5). It was reported that this endonuclease sensitive site was not an apurinic or apyrimidinic site since heavily irradiated PM 2 DNA was not alkali-labile (7). However, we found that under conditions of alkaline hydrolysis used by Lindahl and Andersson for detection of apurinic sites (8), alkali-labile sites are present in UV irradiated DNA.

Methods. H^3-thymidine labeled PM 2 DNA with a specific activity of 2×10^4 DPM·μg^{-1} was prepared as previously

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described (2, 3) and irradiated with a 15W GE germicidal bulb at an incident energy of 108 ergs/mm^2.sec^{-1} as measured with a Latarjet meter. Pyrimidine dimer content was measured by paper chromatography (9) and the number of breaks in DNA estimated from the Poisson distribution after sedimentation velocity centrifugation (2, 3).

For determination of alkali-labile sites after UV irradiation, the following hydrolysis protocols were used: A) 4 hours in 1.0M glycine-NaOH, pH 12.8 at room temp. (8); B) 20 minutes in 0.1N NaOH at 37 C (5, 10); or C) 2 hours in 0.3M $K2HPO_4$, pH 12.3 at room temp. (7).

The number of alkali-labile sites appearing after A or B was determined by alkaline sedimentation velocity gradients (3) and after C by neutralization and filtration through nitrocellulose filters to which nicked DNA sticks (7). Breaks were estimated by the Poisson distribution.

In a separate experiment, DNA was irradiated for 3 minutes, incubated at neutral pH for 3.5 hours and then hydrolyzed by methods B and C.

Heat induced apurinic sites were introduced as previously described (3).

Partially purified Uracil-N-glycosidase and apurinic/apyrimidinic DNA endonuclease were prepared from sonicated HeLa cells. After centrifugation at 20,000 g x 15 min., the sonicate was passed through a DEAE-23 column, pH 8.0, 10mM potassium phosphate, 1mM EDTA, 0.5mM DTT, then adjusted to pH 7.5 and placed on a Whatman P-11 phosphocellulose column and eluted with a linear gradient of KCl from 0.01M to 0.6M, 10mM potassium phosphate, 1mM EDTA, 0.5mM DTT, pH 7.5. The fractions containing Uracil-N-glycosidase activity and apurinic/apyrimidinic activity were pooled separately and placed on hydroxylapatite columns, 10mM potassium phosphate, 0.15M KCl, 1mM EDTA, 0.5mM DTT, pH 7.2. Glycosidase activity was eluted with 0.1M potassium phosphate and apurinic/apyrimidinic endonuclease activity with 0.2M potassium phosphate. Uracil-N-glycosidase activity was assayed using H^3-uridine labeled PBS DNA (11) and apurinic/apyrimidinic endonuclease activity by a filter method (5), gradient analysis (2, 3) and by combination with glycosidase using PBS DNA (12). These enzyme activities were tested against UV irradiated PM 2 DNA singly and in combination and the nicks in DNA assayed by filter method or gradient analysis (2, 3, 5). One unit of Uracil-N-glycosidase releases one pmole of uracil/minute under the conditions employed. One unit of apurinic endonuclease introduces one break per PM 2 molecule/10 minutes in DNA containing 1-2 apurinic sites/molecule. One such endonuclease unit, coupled with 5 units of glycosidase released 25% additional radioactivity from PBS H^3-labeled

DNA which was not free uracil.

Results. Dimer production was non-linear with 3.5, 5.5 and 6.4% of radioactivity in dimers appearing at 1, 2 and 3 minutes of irradiation. No nicks were introduced by this procedure.

To show that all three methods of hydrolysis are equally effective in detecting apurinic sites, alkaline hydrolysis was performed on aliquots of heated, acidified DNA. The results are shown in Table I and indicate that all 3 methods yield similar results.

TABLE I
COMPARISON OF THREE METHODS FOR ESTIMATION OF APURINIC SITES

Method	Minutes of Heat	Alkali-labile sites/PM 2 Molecule[a]
A	0	0.11
	5	0.48
	10	0.82
B	0	0.11
	5	0.39
	10	0.82
C	0	0.09
	5	0.50
	10	1.02

[a]Control background value subtracted from experimental determinations.

When UV irradiated DNA was hydrolysed, only method A detected the linear production of alkali-labile sites showing that approximately 0.50 sites/molecule were introduced for each minute of irradiation. Hydrolysis by B and C yielded only 0.20 additional sites per molecule after 3 minutes of irradiation.

However, when DNA was incubated for 3.5 hours at neutral pH at room temp. after UV irradiation, an increased number of alkali-labile sites were detected by both methods B and C.

To show that these sites which appear are indeed apurinic or apyrimidinic sites, this DNA was incubated with purified apurinic/apyrimidinic endonuclease, with purified Uracil-N glycosidase and a combination of the two activities. The results are shown in Table II.

Discussion. We conclude from this data that UV irradiation (254nm) causes at least two types of alkali-labile sites in DNA. One is an apurinic and/or apyrimidinic site that is initially formed in very small amounts and is barely detectable by methods B and C. This is consistent with the

TABLE II
NICKS INTRODUCED INTO UV IRRADIATED PM 2 DNA BY HELA ENZYMES

Time Post UV[a]	Enzyme[b]	Nicks/Mol.	Alk.lab.site/Mol.[c]
0	AP endonuclease	0.19	0.18
0	N-glycosidase	0.05	0.18
0	Both	0.15	0.18
3.5 hrs	AP endonuclease	0.34	0.43

[a]DNA irradiated for three minutes (19,500 ergs/mm^2).
[b]5 units of glycosidase, 1 unit of endonuclease.
[c]As determined by methods B and C.

demonstration by ourselves and others that purified apurinic endonucleases do not attack UV irradiated DNA (5, 12, 13). Hydrolysis by method A revealed additional alkali-labile sites. The possibility that these are unstable bases is indicated by the increase in apurinic/apyrimidinic endonuclease sensitive sites after 3.5 hours of incubation of the irradiated DNA. This corroborates previous reports of increased sensitivity to apurinic endonucleases of aged or heated UV irradiated DNA (5, 13). Our data indicate that the putative unstable base, detectable only by method A, is formed at a ratio of about 0.08 sites per molecule for each 1000 ergs/mm^2 of irradiation. This figure is in good agreement with the production of non-dimer endonuclease sensitive sites reported by others which range from 0.05 sites/molecule using HeLa cell (1) or rat liver (3) preparations, to 0.12 sites for E. coli Endonuclease III (7) and 0.17 sites for an endonuclease from calf thymus (4). Hydrated thymines of the 5.6, dihydroxydihydrothymine type are formed in a similar amount in DNA by 254nm irradiation (14) and may represent the actual target of these UV non-dimer endonucleases. To the best of our knowledge, the relative alkaline stability of these hydrated thymines is not yet known.

It is also apparent that the UV non-dimer endonuclease activity of HeLa cells is not a composite activity of Uracil-N-glycosidase and the apurinic/apyrimidinic endonuclease as shown by the results in Table II.

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PARTIAL PURIFICATION AND CHARACTERIZATION OF THREE THYMINE DIMER EXCISING ACTIVITIES FROM HUMAN KB CELLS[1]

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ABSTRACT Three thymine dimer excising activities previously reported by Cook and Friedberg (1978) have been further purified and characterized.

INTRODUCTION

Our laboratory has been actively investigating enzymes from mammalian cells which are capable of excising thymine-containing pyrimidine dimers (dimers) from DNA (1,2). The existence in *E. coli* of at least four distinct enzymes capable of 5' → 3' excision of dimers *in vitro* (See ref. 3), none of which is uniquely responsible for *in vivo* excision (4,5), suggests that this situation may be mirrored in eukaryotes as well. There have been two reports of exonucleases isolated from mammalian cells which are capable of 5' → 3' excision of dimers from purified DNA: DNase IV, reported by Lindahl, *et al.* (6) and "correxonuclease", reported by Doniger and Grossman (7). These activities were identified and partially purified using as an assay the non-specific degradation of poly d(A-T) and UV-irradiated denatured DNA, respectively.

We have approached the problem of identifying dimer excising nucleases by using a direct assay of the selective release of dimers from UV-irradiated DNA previously incised 5' to dimers with the pyrimidine dimer specific endonuclease of phage T4 (endonuclease V) (8). This approach makes the assumption that the T4 endo. V nicked, UV-irradiated DNA is a reasonable analog of the *in vivo* substrate. The advantages of this approach are the ability to assay activities which may not degrade less specific DNA substrates and the elimination of activities which

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may randomly degrade UV-irradiated DNA, but which may not excise dimers specifically from preincised double-stranded DNA. We have pursued this approach using mammalian cells grown in tissue culture because of the advantages of controlled growth conditions and homogeneity of cell type.

RESULTS

A scheme for the partial purification of 3 dimer excising nuclease activities starting with cytoplasmic extracts of human KB cells has been reported (2). We have continued to purify and study these activities. The behavior of each activity through four steps of purification is shown in Table I. Each activity behaves in a unique manner through these four purification steps, and maintains its character upon rechromatography.

A preliminary characterization of activities A, B and C has been previously reported (2). Activity B is physically quite distinct because of its small size (27,000 MW) and its high isoelectric point (9.0). Activities A and C appear physically to be quite similar. Both have an isoelectric point of about 6.0 and a sedimentation value of 3.0 - 3.2 S. However, non-denaturing polyacrylamide gel electrophoresis of the 3 activities results in a different mobility for each, although the yields from the gels have thus far been poor. In addition, the response of the 3 activities to various concentrations of divalent cations, monovalent chloride salts and p-chloromercuriphenyl sulfonic acid is distinct (2).

The characterization of the substrate specificity of each activity is complicated by the fact that we do not yet have homogeneous proteins. We have, however, examined their effects on various substrates as is summarized in Table II. All three activities can excise thymine-containing pyrimidine dimers from preincised duplex DNA, but none degrades unincised double-stranded DNA. Activity A is distinguished by its inability to degrade poly d(A-T)whereas activity B does not significantly degrade denatured DNA to acid-soluble products. Both activities A and C do degrade this substrate irrespective of the presence of photoproducts in the DNA. Activity C clearly is, or has

TABLE I

SEPARATION CHARACTERISTICS OF
KB DIMER EXCISING ACTIVITIES

Purification Step	Activity A	Activity B	Activity C
0.2 M Acetate, pH 5.5	soluble	soluble	insoluble
[NaCl] to elute from phosphocellulose (molar)	0.0	0.15	0.25
[NaCl] to elute from DEAE cellulose (molar)	0.11	0.06	0.08
[PO_4] to elute from hydroxylapatite (molar)	0.0	0.04	0.05

TABLE II

ABILITY OF KB DIMER EXCISING ACTIVITIES TO DEGRADE VARIOUS SUBSTRATES

Substrate	Activity A	Activity B	Activity C
Preincised UV-irradiated DNA	Selective excision of dimers	Selective excision of dimers	Selective excision of dimers
Unincised UV-irradiated double-stranded DNA	No	No	No
Unirradiated double stranded DNA	No	No	No
UV-irradiated single stranded DNA	Yes	Very little	Yes
Unirradiated single stranded DNA	Yes	Very little	Yes
Circular single stranded DNA	No	No	Yes
Supercoiled double stranded DNA	No	No	Nicks
poly d(A-T)	No	Yes	Yes

associated single strand endonuclease activity and also nicks supercoiled PM2 DNA (possibly recognizing the single-stranded regions known to exist in this substrate).

DISCUSSION

The demonstrated ability of a number of enzymes from uninfected and phage T4-infected *E. coli* to excise dimers *in vitro* (see ref. 3) suggests that this activity may reflect a relative lack of substrate specificity in enzymes whose primary function *in vivo* may be quite different. This may in part account for the detection of multiple activities in extracts of human cells in culture. On the other hand, it remains distinctly possible that redundancy for this function is biologically significant.

Our studies to date suggest that there are 3 major nuclease activities present in cytoplasmic extracts of human cells, capable of selective removal of dimers from UV-irradiated DNA incised with T4 endonuclease V. There may also be other activities present in low amounts, or which under our assay conditions are not particularly efficient at selective dimer removal. When our activities are compared to DNase IV and "correxonuclease" certain similarities are evident. Activity C has similar affinities for ion-exchange matrices and a size similar to that of "correxonuclease". However, to date we have not been able to unambiguously identify activity C as a single strand exonuclease rather than an endonuclease (see below). Activity B bears a certain similarity to DNase IV, since both do not actively degrade denatured or native DNA and do degrade poly d(A-T). However, activity B is significantly smaller than DNase IV and is optimumly stimulated by Mg^{++} rather than Mn^{++}.

The detailed characterization of these enzymes presents interesting problems in substrate specificity and in nomenclature. It has been generally assumed that enzymes that ex-

cise thymine dimers are necessarily exonucleases. However, this function may be catalyzed by endonuclease activities that recognize the single-stranded nature of the dimer-containing sequence generated by previous incision of the DNA and that do not specifically require a free end. Thus, for instance, we have demonstrated dimer excision from preincised DNA with the S_1 nuclease from *Aspergillus oryzae* (K. H. Cook and E. C. Friedberg, unpublished observations). It should also be noted that a number of laboratories have reported the purification of single strand specific endonucleases from mammalian cells, but it is not known whether these catalyze excision of thymine dimers from preincised UV-irradiated DNA (9-11).

As regards nomenclature, some enzymes may contain both endonuclease and exonuclease activities. An instructive example is the *recBC* nuclease of *E. coli*, which degrades single-stranded DNA endonucleolytically and both single and double-stranded DNA exonucleolytically from either 3' or 5' ends (12). Recently it has been reported that homogeneous *Crotalus adamanteus* venom phosphodiesterase has an associated endonuclease activity specific for single-stranded and superhelical DNA (13). These examples suggest that the demonstration of both endonuclease and exonuclease activities in a single fraction, does not necessarily imply the presence of more than one enzyme. In our hands, the currently most purified fraction of activity C definitely contains both dimer excising and single-stranded endonuclease activity. The ability of this fraction to also degrade single-stranded linear DNA may be indicative of an additional exonuclease activity,but this need not be a contaminant. Clearly it is necessary to further purify and characterize these activities to resolve these questions.

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INTRODUCTION OF T4 ENDONUCLEASE V INTO FROZEN AND THAWED MAMMALIAN CELLS FOR THE DETERMINATION OF REMOVAL OF UV INDUCED PHOTOPRODUCTS[1]

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ABSTRACT The reduction of DNA single strand molecular weight by T4 endonuclease V has been used to evaluate removal of pyrimidine dimers in mammalian cells in which the endonuclease is introduced directly through permeabilization of the cellular membranes by two cycles of rapid freezing and thawing. In V79 Chinese hamster cells the number of nicks introduced by T4 endo is a linear function of UV dose measured up to 5 J/m^2. Comparisons of the molecular weights so obtained with the number of pyrimidine dimers, measured chromatographically, show that about 50% of the dimers are T4 endo sensitive sites in this assay. DNA isolated from permeabilized cells exposed to T4 endo was shown to contain the expected number of additional sites. The method described has also been used to show that T4 endo sites do occur in DNA synthesized at early times following UV irradiation, but not at late times.

INTRODUCTION

UV specific endonuclease has been used to evaluate removal of pyrimidine dimers in bacterial systems in which the endonuclease is introduced directly into detergent-permeabilized cells (1). In studies with mammalian cells the technique has been applied to isolated DNA, but it is limited by the difficulty of isolating DNA of very high molecular weight (2). This paper describes a technique for introducing T4 endonuclease V into mammalian cells in order to determine the frequency of UV-induced pyrimidine dimers.

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MATERIALS AND METHODS

V79 Chinese hamster cells were labeled for at least one cell generation with ^{3}H-thymidine ([.25-.5 Ci/mMole], 2 μCi/ml) or with ^{14}C-thymidine (50 Ci/Mole, 0.1 μCi/ml). For pulse labeling, ^{3}H-thymidine with a specific activity of 40-60 Ci/mMole was used.

T4 Endonuclease V Assay. Cell suspensions (2.5×10^{6} cells per ml) were prepared in 0.06 M tris, pH 8, 0.01 M EDTA, 0.1 M NaCl, 0.01 M β-mercapto ethanol and 1 mg/ml bovine serum albumin. 80 μl samples were twice rapidly frozen and thawed using a solid CO_2-ethanol mixture. After incubation with 10-20 μl T4 endo for 15 minutes at room temperature, 50 μl was lysed for 2.5-3 hours on top of an alkaline sucrose gradient (3). Centrifugation was for 110 minutes at 30,000 rpm (20° C). Number average molecular weights were determined using phage T2 as marker (4).

DNA Isolation and Pyrimidine Dimer Assay. Cells were lysed with 0.5% sarcosyl in 0.01 M tris, pH 8 and 0.01 M EDTA and incubated overnight with 50 μg/ml proteinase K at 37° C. DNA was purified from these lysates by centrifugation through CsCl (1.4 g/ml) to a shelf (1.8 g/ml). The DNA collected from the interphase of the two CsCl layers was dialyzed against 0.01 M tris, 0.01 M EDTA and 0.1 M NaCl, pH 8. The frequency of pyrimidine dimers was determined by chromatography of acid hydrolysed DNA as described (5, 6).

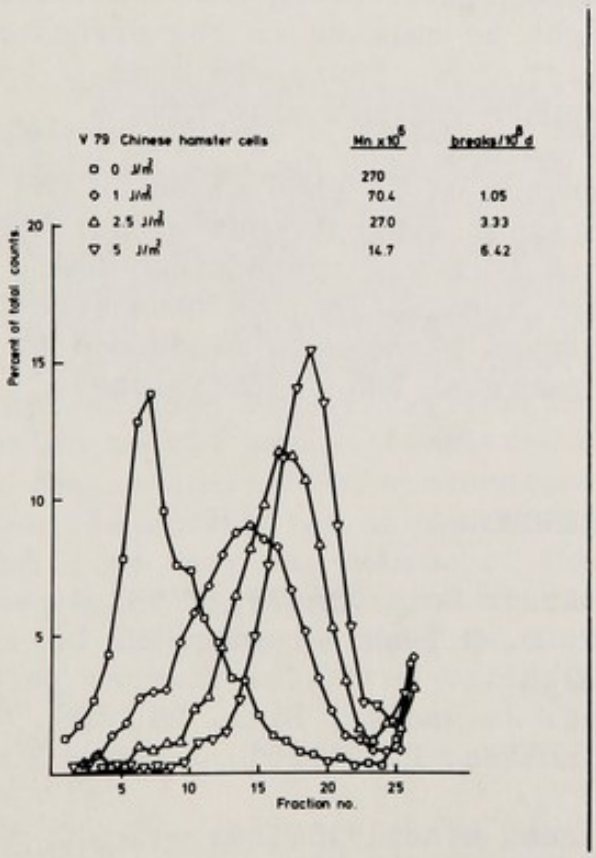
RESULTS

In order to treat DNA with T4 endo inside cells, the cellular membrane must be permeable to the enzyme. Rapid freezing and thawing of a cell suspension renders the DNA accessible to T4 endo. The reduction in molecular weight of the DNA was determined by sedimentation of DNA of cells lysed directly on alkaline sucrose gradients. Figure 1 shows an effect of T4 endo on the size of DNA from freeze-thaw irradiated cells. A dose as low as 1 J/m^{2} causes a large shift in the gradient. All cells became permeable since at higher doses no DNA of control size was seen. Experiments with unirradiated cells showed that rapid freezing and thawing does not nick the DNA and that T4 endo does not nick dimer-free DNA. The frequency of T4 endo sites increased linearly with UV dose with a slope of 1.24 sites per 10^{8} dalton per J/m^{2}. However the frequency of pyrimidine dimers, determined chromatographically, increased with a slope of 2.42 dimers per 10^{8} daltons per J/m^{2}, suggesting that only 50% of the dimers are sensitive to the T4 endo. Upon further analysis of this result, experiments

TABLE 1
ALKALINE SUCROSE CENTRIFUGATION OF DNA TREATED WITH T4 ENDO INSIDE AND/OR OUTSIDE THE CELL (UV-DOSE: 5J/M^2)

Procedure for T4 endo treatment	Mnx10^6	T4 endo sites/10^{8}d
Inside - direct lysis	13.6	7.0
Inside - DNA isolation	14.4	6.6
Inside - DNA isolation - outside	6.6	10.2
DNA isolation - outside	5.1	14.6
No UV - direct lysis	268	-
No UV - DNA isolation	20.0	-
Expected frequency	-	12.1

with DNA isolated from T4 endo treated cells showed that in a second treatment, outside the cell, the additional 50% of the dimers become available as substrate (Table 1).



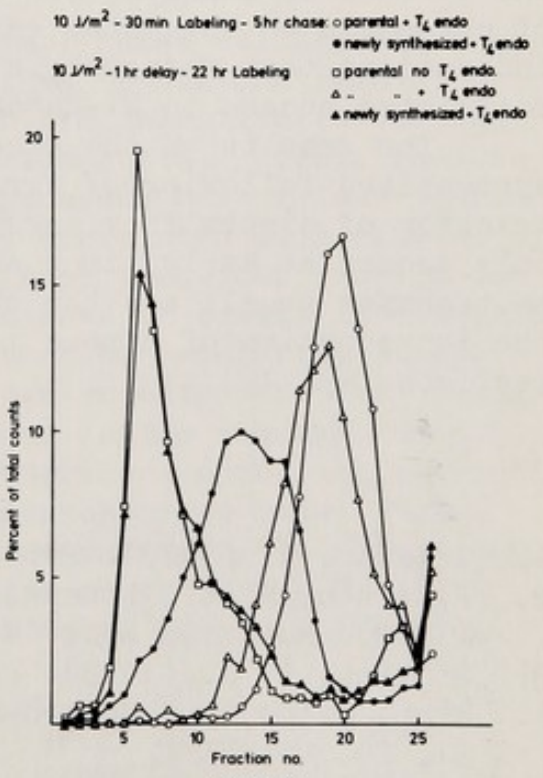


FIGURE 1. Alkaline sucrose gradients of UV-irradiated V79 cells, permeabilized and treated with T4 endo.

FIGURE 2. Alkaline sucrose gradients of V79 cells pre-labelled with ^{14}C-thymidine. After UV irradiation the cells were labelled with ^{3}H-thymidine and treated with T4 endo as indicated.

Use of this T4 endo sensitive site assay to follow excision of dimers, V79 cells exposed to 5 J/m^2 UV showed a small but reproducible loss of sites (26% in 24 hr).

The method was also used to determine the occurrence of T4 endo sites in DNA synthesized at different times after a UV dose of 10 J/m^2. It was found that DNA synthesized during the first 5.5 hr after irradiation does contain T4 endo sites, but that there are no sites detectable in DNA synthesized in a 22 hr period starting 1 hr after irradiation (fig. 2).

DISCUSSION

Rapid freezing and thawing of mammalian cells makes DNA accessible to UV specific endonuclease from phage T4. Since the cells are lysed directly on alkaline sucrose gradients, no DNA isolation step is used, resulting in a high sensitivity of the method. UV doses lower than 1 J/m^2 can be detected. The reason for only 50% of the dimers constituting substrate for the T4 endo is unknown, but might be related to the structure of chromatin as suggested earlier (7). There are some indications that endogeneous repair enzymes also have a restricted access to UV-damage in DNA (8).

The results on the occurrence of T4 endo sites in DNA synthesized following UV irradiation suggest that either the transfer of dimers from parental to newly synthesized DNA only occurs at early times after irradiation, or that there is no transfer at all and the observed phenomenon is caused by the introduction of dimers in parental DNA of unfinished replicons (9, 10).

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REMOVAL OF T4 ENDONUCLEASE V SENSITIVE SITES AND REPAIR REPLICATION IN CONFLUENT HUMAN DIPLOID FIBROBLASTS[1]

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ABSTRACT Following irradiation with low doses of ultraviolet light, the timecourse of removal of DNA sites sensitive to T4 endonuclease V in contact inhibited human diploid fibroblasts (WI38) is in agreement with the timecourse of DNA repair replication in these cells. Repair patches synthesized 12-16 hr after irradiation are of the same size as those made from 0-2 hr.

INTRODUCTION

Following UV irradiation, normal human cells exhibit both repair replication of short stretches of DNA and removal of pyrimidine dimers, phenomena which are defective to varying degrees in most xeroderma pigmentosum cells. Recent measurements of the rates of these two processes have raised doubts that repair replication is exactly correlated with removal of dimers (1). Rate comparisons of these processes usually depend on their endpoints. Complete loss of dimers is a natural endpoint, but repair replication must be considered in relation to the amount completed after an extended time. Changes in the amount of DNA synthesized per lesion removed, in the specific activities of radioactive tracers used, and in cell viability over the course of the measurements might hinder meaningful interpretation of such comparisons. The study reported here was designed to minimize these difficulties by using contact inhibited cells irradiated with low UV doses.

RESULTS

Repair replication in contact inhibited WI38 cells was determined by incorporation of ^{3}H-BrdUrd (2). The lengths of the DNA stretches synthesized were determined by sonicating isolated parental density DNA and centrifuging in alkaline CsCl gradients in a swinging bucket rotor (3). The shifts in

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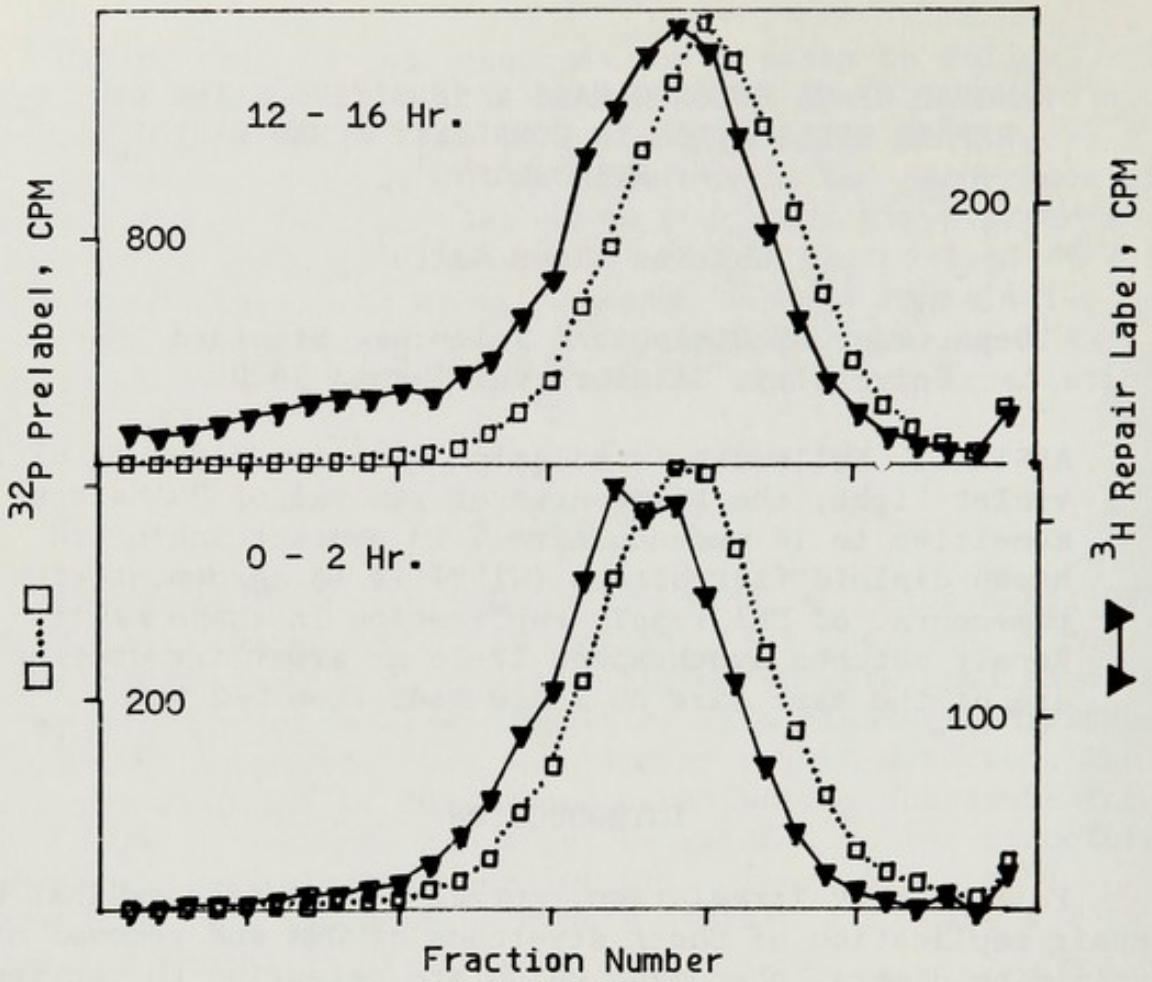

FIGURE 1. Profiles of alkaline CsCl gradients of sonicated parental density DNA from cells labelled with ^{3}H-BrdUrd for the times indicated after 10 J/m^{2} UV. Field is to the left

density of the fragments labelled during repair replication with ^{3}H-BrdUrd with respect to that of the bulk ^{32}P-DNA was about 13% of that for fully BU-substituted hybrid DNA for both 0-2 and 12-16 hr post UV intervals (Fig. 1). Since the average fragment length was about 250 nucleotides, the "patch size" made in both intervals was about 30 nucleotides. Four times as many cells were used for the 12-16 hr interval and residual hybrid DNA not removed by the initial neutral CsCl gradient probably accounts for the small amount of highly shifted DNA in that case. The average rate of repair replication from 12-16 hr post UV was 21% of that from 0-2 hr.

Repair replication was next measured in cells incubated for various lengths of time after irradiation, up to 48 hr (Fig. 2). The contact inhibited unirradiated cells incubated for 24 hr replicated only about 5% of their DNA, judged from the ^{3}H incorporation into hybrid DNA. In this experiment, the average rate of repair replication from 12-16 hr post UV was 20% of that from 0-2 hr.

Accurate determination of pyrimidine dimer content in DNA

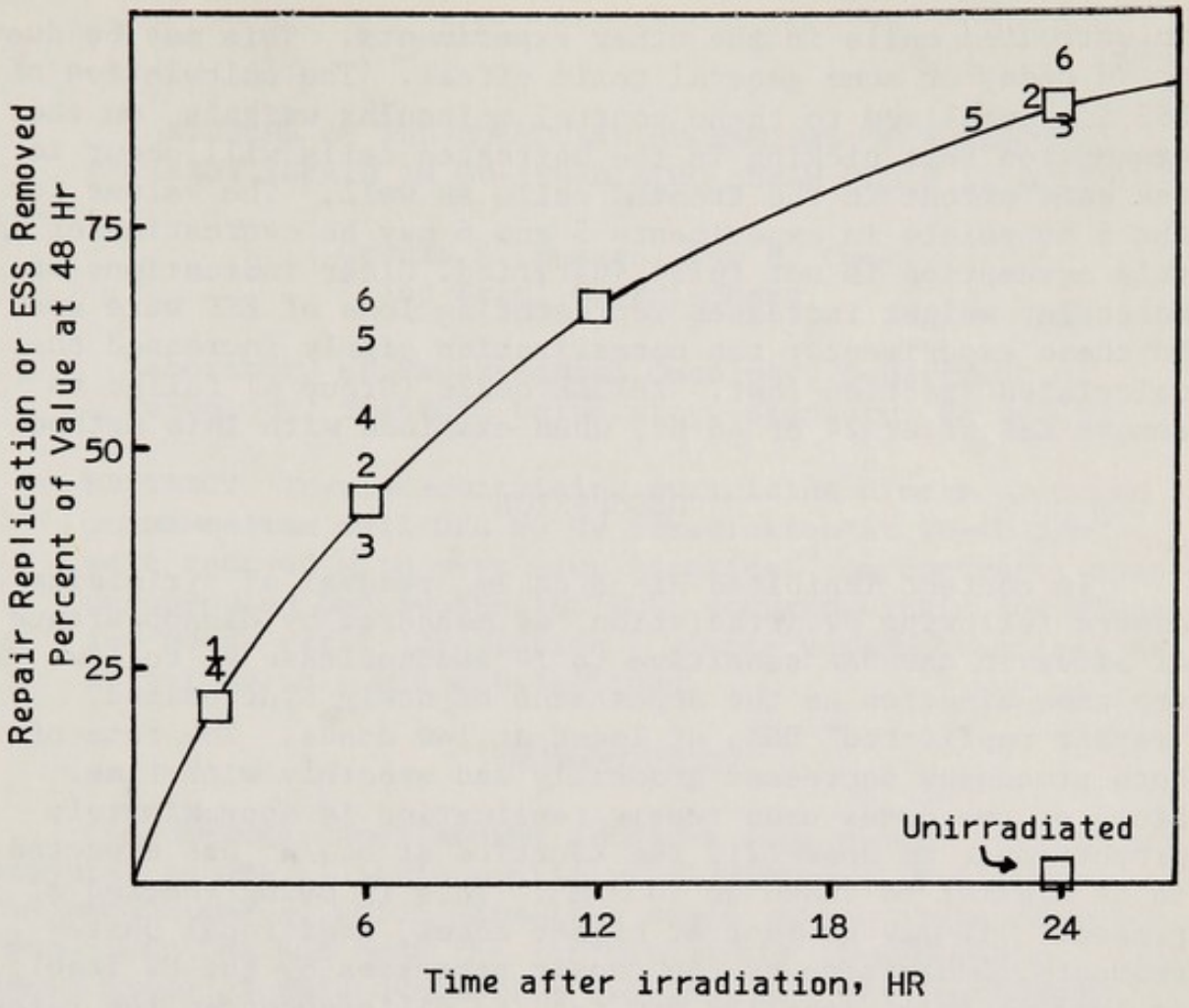

FIGURE 2. Repair replication (□, ^{3}H incorporated/unit DNA) *vs.* time after 10 J/m^2 and fraction of ESS removed (numbers, each representing an experiment) after 5 J/m^2.

irradiated at these low doses is difficult and requires either large quantities of cells or cells labelled with high concentrations of radioisotopes, which have toxic effects. Sites in the DNA which are sensitive to nicking by the pyrimidine dimer specific T4 endonuclease V (ESS) correlate well with pyrimidine dimers and are easily measured in cells irradiated with 5 J/m^2 UV (4). The removal of ESS in irradiated WI38 cells was measured by incubating frozen and thawed cells with the enzyme and determining DNA molecular weights with alkaline sucrose gradients (4). About 85% of the ESS were removed in 48 hr and data from several experiments were normalized to this value for comparison to the repair replication data (Fig. 2). In experiments 1-4, where the cells grew normally to confluence in low specific activity ^{14}C- or ^{3}H-dThd, the data agree well with the repair repliaction data. In experiments 5 and 6, the cells were labelled with a toxic level of ^{3}H-dThd (5 μCi/ml, 50 Ci/mMole), and the molecular weights of DNA from cells not exposed to the endonuclease were lower than those

in untreated cells in the other experiments. This may be due to ^{3}H decay or some general toxic effect. The calculation of ESS is normalized to these control molecular weights, on the assumption that nicking in the untreated cells will occur to the same extent in the treated cells as well. The values for the 6 hr points in experiments 5 and 6 may be overestimates if this assumption is not fully justified. Clear indications of molecular weight increases representing loss of ESS were seen in these experiments; the normalization simply increased the calculated fraction lost. XP12BE cells (Group A) failed to remove ESS after 24 or 48 hr, when examined with this method.

DISCUSSION

In contact inhibited WI-38 cells, removal of pyrimidine dimers following UV irradiation, as measured by disappearance of sites in the DNA sensitive to T4 endonuclease V, follows the same kinetics as the appearance of newly synthesized, "repair replicated" DNA, at least at low doses. The rate of both processes decreased gradually and smoothly with time. Since at the doses used repair replication is approximately proportional to dose (2), the kinetics at 5 J/m^2 are expected to be similar to those at 10 J/m^2. This is being checked at present. It may be that at higher doses, additional photoproducts, inhibition of the repair processes by the UV itself, or loss in cell viability may lead to differences in the rates of repair replication and photoproduct removal.

ACKNOWLEDGEMENTS

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STUDIES ON THE MOLECULAR MECHANISMS OF NUCLEOTIDE EXCISION REPAIR IN UV-IRRADIATED HUMAN CELLS IN CULTURE[1]

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ABSTRACT Thymine-containing pyrimidine dimers produced in mammalian cell DNA by UV irradiation at 10-40 J/m^2 were removed with very slow kinetics. In contrast, most unscheduled DNA synthesis (UDS) occured within the first few hours after irradiation. Dimers were not excised as acid-insoluble oligonucleotides.

INTRODUCTION

Cyclobutane dipyrimidine products (pyrimidine dimers) are produced in DNA of living cells by ultraviolet (UV) light. The mechanisms by which mammalian cells remove these products from their DNA has been the subject of our investigation. The basic steps of this nucleotide excision process are thought to be similar to those in bacteria. Endonucleolytic incision of the DNA strand near the dimer is followed by excision of an oligonucleotide containing the dimer as well as by repair synthesis. The relative order of the last mentioned events in mammalian cells is not known, and we have measured the kinetics of these events in an attempt to clarify their relationship.

MATERIALS AND METHODS

Cells and Cell Growth: The following cell types were used: GM38 and WI38, normal human diploid skin fibroblast lines; VA13, an SV40 transformed WI38 cell line; human KB cells (a tumor cell line) xeroderma pigmentosum skin fibroblast lines XP12BE (Jay Tim, complementation group A) and XP5BE (BeWen, complementation group D); two xeroderma pigmentosum variant skin fibroblast lines, XP4BE (WoMec) and XP13BE (PeHay); and

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the Fanconi's anemia skin fibroblast line HG261. All cells were grown in Eagle's Modified Essential Medium supplemented with 10% fetal bovine serum as described elsewhere (1) and all experiments were performed with cells at passages 10-30.

Measurement of thymine dimer excision *in vivo*. Cells were plated in petri dishes 3 days before irradiation. After irradiation with UV light, cells were incubated for various times before precipitation with cold 10% trichloroacetic acid (TCA) (1). In some cases nuclei or chromatin (2-4) were isolated before TCA precipitation (1). The TCA precipitates were hydrolyzed and dimer contents determined chromatographically as described elsewhere (1). In some experiments the high molecular weight DNA (> $\sim$ 5 x 10^6 daltons) were separated from DNA of smaller size on alkaline sucrose gradients (1). The large DNA was then precipitated with 10% TCA for dimer assays (1).

Unscheduled DNA Synthesis. Unscheduled DNA synthesis was measured by autoradiography. Cells grown on coverslips were pulse labelled at different times after irradiation with ^{3}HTdR. The coverslips were affixed to slides, dipped into autoradiographic emulsion and developed as described elsewhere (1). Grains were counted microscopically.

RESULTS

The removal of thymine dimers from cellular DNA was a relatively slow process. Very little, if any, dimer loss was apparent within the first 5-6 hours after irradiation (fig. 1A). The number of dimers decreased thereafter until 24-30 hours after irradiation, after which time no additional dimer loss occured. This pattern was observed in all the cell types examined except cells from xeroderma pigmentosum groups A and D, in which no dimer loss was detected. The results for GM38 cells in the experiment of fig. 1A is, therefore, typical of all the results obtained at fluences between 10-40 J/m^2. In some experiments (data not shown, ref. #1) the kinetics of dimer removal were followed up to 72 hours after irradiation, and no additional dimer loss was seen. The time at which about half of the removeable dimers were lost was 17 hours in this experiment and ranged from 10-22 hours in different experiments (1). The same kinetics were observed when dimer measurements were performed on isolated nuclei (fig. 1a) or chromatin (J. I. Williams and E. C. Friedberg, unpublished observations). We also observed that at fluences between 10-40 J/m^2 the absolute calculated number of thymine dimers lost from cells was dose independent, i.e., the higher the fluence the smaller the percentage of UV-induced dimers removed.

In order to be sure that the apparently slow rate of dimer

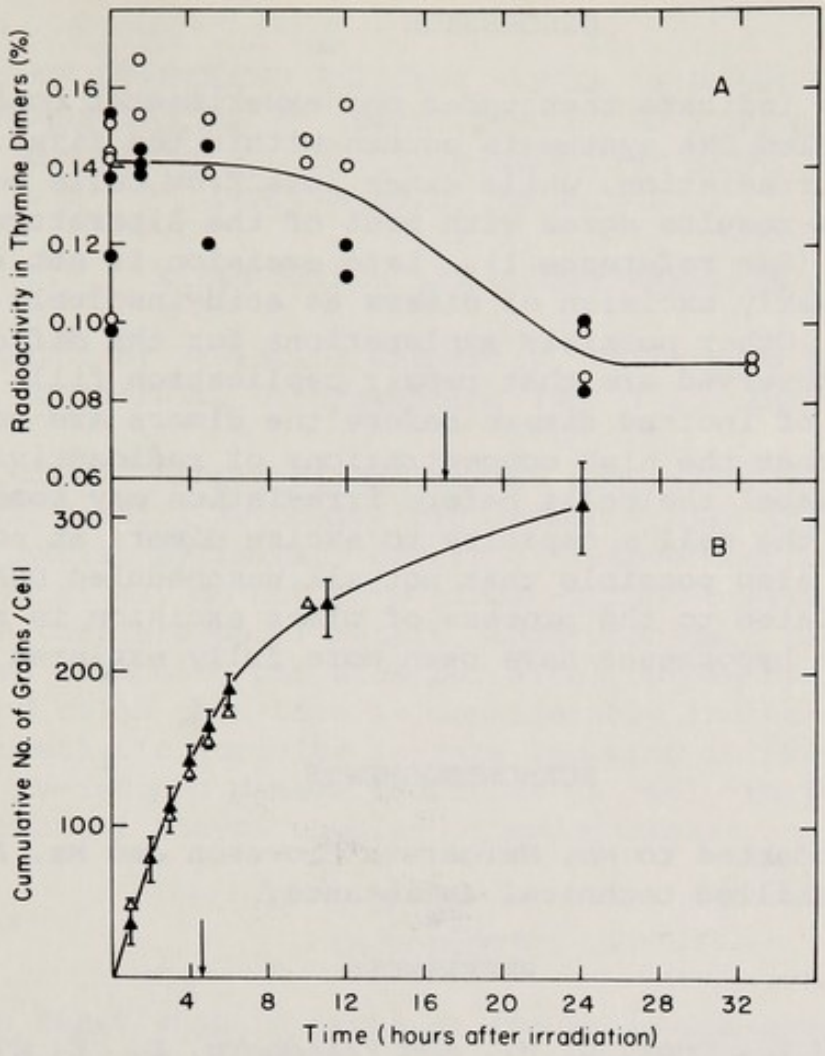

FIGURE I. The kinetics of dimer excision and unscheduled DNA synthesis in GM38 cells. A. Thymine dimer removal after 20 J/m^2 UV light. 0, whole cell TCA precipitates; ●, TCA precipitates of isolated cell nuclei. B. Unscheduled DNA synthesis after 15 ▲, or 40 Δ J/m^2. Arrows indicate the time at which half of the repair was completed.

loss was not an artifact of measuring the dimer content of the acid-precipitable DNA (which would have included the excised oligonucleotides if they were greater than ∿ 15 nucleotides in length), we also performed dimer assays on purified DNA > ∿ 5 x 10^6 daltons in molecular weight. This DNA was separated by alkaline sucrose sedimentation. The kinetics of dimer loss was the same when measured on this fraction of DNA as when measured with whole cell precipitates (1).

When unscheduled DNA synthesis was measured in GM38 cells irradiated with 15 or 40 J/m^2, most of the incorporation of label occurred during the phase when little, if any, dimer loss was observed. Half of the total amount of repair which occurred within the 24 hours of the experiment occurred within about the first 4 1/2 hours (fig. 1B).

DISCUSSION

Our data indicate that under our experimental conditions, most unscheduled DNA synthesis occurs within the first few hours after irradiation, while dimer loss from cells occurs later. These results agree with most of the literature on this subject (See reference 1). Late excision is not an artifact of the early excision of dimers as acid-insoluble oligonucleotides. Other possible explanations for the differences in kinetics observed are that repair replication fills in gaps at the sites of incised dimers before the dimers are actually excised, or that the high concentrations of radioactivity which are used to label the cells before irradiation may somehow interfere with the cell's capacity to excise dimers at normal rates. It is also possible that not all unscheduled DNA synthesis is related to the process of dimer excision in mammalian cells. These hypotheses have been more fully explored elsewhere (1).

ACKNOWLEDGMENTS

We are indebted to Ms. Margareta Brorsson and Ms. Adriana Johnson for skilled technical assistance.

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KINETICS OF UV-INDUCED EXCISION REPAIR IN HUMAN FIBROBLASTS.[1] SIMULTANEOUS STUDIES OF INCISION-PRODUCED SINGLE-STRAND BREAKS AND THE REMOVAL OF ENDONUCLEASE-SENSITIVE SITES USING THE DNA UNWINDING TECHNIQUE .

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ABSTRACT Single-strand breaks are produced during excision repair. Xeroderma pigmentosum cells that are unable to excise pyrimidine dimers or remove endonuclease-sensitive sites, do not form single-strand breaks. Sites in DNA that are under repair stand upon as a nick for 3 minutes (assuming one nick per site) but in hydroxyurea-treated cells this time is considerably increased. The endonucleolytic activity is rate limiting in excision repair of UV-induced damage and it is unlikely that there is a permanent enzyme complex of endonuclease and polymerase.

INTRODUCTION

The first step in excision repair of DNA exposed to ultraviolet light (UV) appears to be a recognition of the defect and incision by a specific endonuclease, giving rise to a single-strand break. In the following reactions, exonuclease and polymerase restores the original sequence of DNA but the site under repair is still associated with a single-strand break until the final phosphodiester bond has been formed by action of the ligase. Thus, the level of strand breaks due to excision repair in a cell, must be a function of the rate with which these nicks are formed and the time they stand open.

A simple and sensitive procedure, the DNA unwinding technique (1, 2, 3) has made it possible to study details of these reactions in human cells.

RESULTS

The DNA unwinding technique has been used to study the level of single-strand breaks (SSB) in cellular DNA formed after UV exposure. The extent and duration of these endonucleolytically formed breaks is dose dependent. Hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase and a potent

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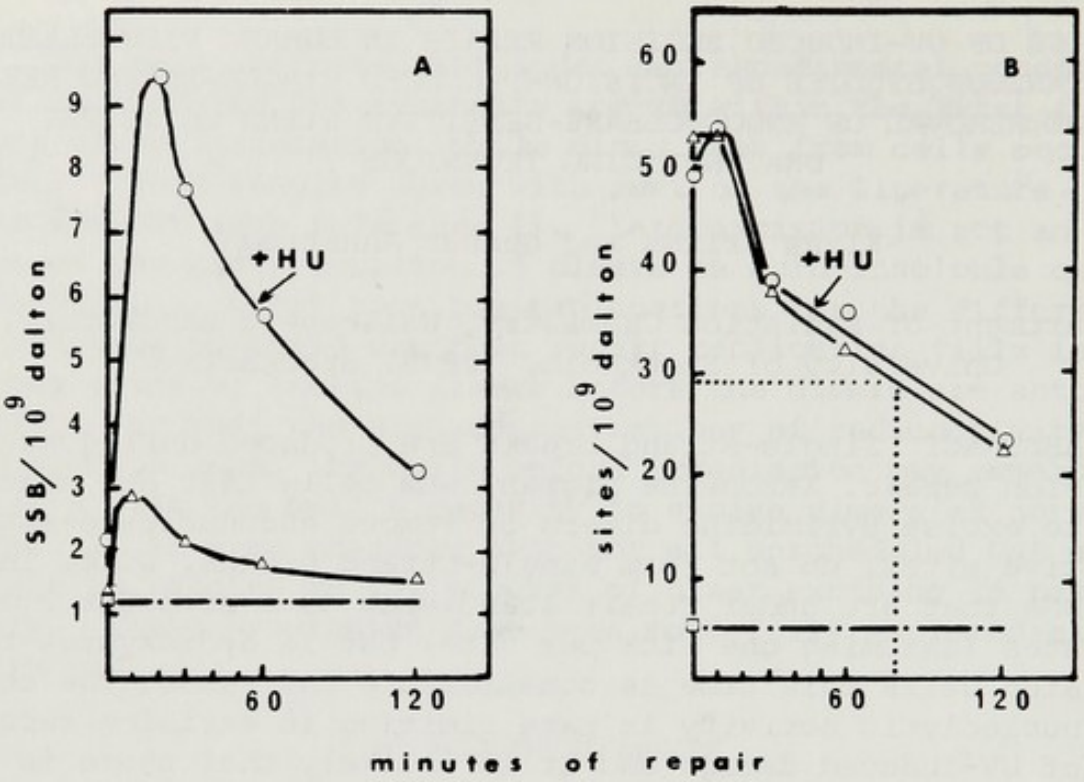

Repair kinetics in normal cells after exposure to UV. A. Internal single-strand breaks (SSB). B. M. luteus endonuclease-sensitive sites as a function of time of repair. (Δ, O) in the absence and presence of 10^{-2} M hydroxyurea. Cells with HU were preincubated for 30 min before irradiation with the inhibitor, (□) no UV. Confluent cell cultures labelled with ^{3}H-thymidine were irradiated with 5 J/m^2 of254 nm light at 0°C. The prewarmed medium was added back and incubation at 37°C was continued for indicated times. For determination of endonuclease-sensitive sites, cells were permeabilized by treating the monolayers with 0.2 per cent Brij in 0.15 M NaCl at 20°C for 5 min. The cells were then incubated with a crude extract of Micrococcus luteus endonuclease for 20 min at 37°C.

The number of single-strand breaks present in the cells were determined by the DNA unwinding technique. Unwinding solution in A: 0.03 M NaOH, 0.15 M NaCl at 20°C for 30 min, B: 0.03 M NaOH at 0°C for 30 min.

inhibitor of DNA synthesis drastically increases the number of breaks observed during the repair process (fig 1a). This is probably due to a decreased rate of repair polymerization, which will cause the enzymatically formed breaks to stand open for longer times. However, in the presence of hydroxyurea, there is no concomitant reduction of repair-induced incorporation of ^{3}H-TdR (4) and no reduction in removal of Micrococcus

luteus endonuclease-sensitive sites (fib 1b).

Integrating the curves in fig 1a for the first 120 min gives 85 and 405 SSB • min/10^9 dalton respectively for cell cultures without and with hydroxyurea. The number of endonuclease-sensitive sites that has been removed during this time is found in fig 1b and corresponds to 28 sites/10^9 dalton. We now assume that each site that is removed will give rise to one and only one SSB. By dividing the obtained integrals with the number of sites removed we can now calculate the time from incision by the endonuclease to final sealing by the ligase to 3 min for the uninhibited cells and 15 min for the hydroxyurea treated cells.

In Xeroderma pigmentosum cells, belonging to complementation group A (XP 12 BE) no breaks were formed, and there was no effect of hydroxyurea. The yield of endonuclease-sensitive sites was the same as for normal cells, about 10 sites/10^9 dalton per J/m^2, but there was no removal of such sites over a period of 4 hours.

DISCUSSION

Hydroxyurea profoundly increases the time required to complete repair, i.e. closure, of individual sites, but has only a marginal effect on the removal of endonuclease-sensitive sites. Our interpretation is that the endonucleolytic activity is the rate limiting event in excision repair of UV-induced damage. Although the rate of nucleotide incorporation at individual sites is drastically decreased, it is still the incision rate that determines the over all rate of excision and repair synthesis. According to this model, there does not seem to be an enzyme complex of endonuclease and polymerase.

If the endonuclease activity is rate limiting in excision repair there should be an agreement with incision rate and excision rate. From fig 1a, only the initial incision rate can be estimated because after 3 or 15 min respectively, the sealing process will interfere. A rough estimate from the plot for hydroxyurea treated cells in fig 1a gives an initial incision rate of more than 0.4 SSB formed per minute in 10^9 dalton. From fig 1b the estimated rate for removal of sites is 0.9 sites/min • 10^9 dalton for the time interval 10-20 min and then 0.17 sites/min • 10^9 dalton, so that after 80 min the number of sites introduced by the dose of 5 J/m^2 has been reduced to half. Rate of incision and excision are therefore in the same range and may correlate in a one to one ratio.

We find a variation in the extent and time course of strand-breaks after a given dose of UV, which seems to depend on the condition of the cell culture. Similarly, there is a

variation in the time course for the removal of Micrococcus luteus endonuclease-sensitive sites, so that the number of sites induced by 5 J/m^2 was reduced to half in a time interval of 30 min to more than 4 hours. This dependency on culture conditions may partly explain the wide range of excision rates reported for human cell lines.

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THE REMOVAL OF UV-INDUCED PYRIMIDINE DIMERS FROM THE REPLICATED AND UNREPLICATED DNA OF HUMAN FIBROBLASTS[1]

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ABSTRACT Excision repair in UV irradiated human fibroblasts has been examined in portions of DNA replicating after irradiation versus those remaining unreplicated. Two approaches, one using a UV-endonuclease to estimate pyrimidine dimers remaining in DNA, the other using density labeling to measure excision resynthesis, indicate that the extent of repair is the same for both replicated and unreplicated DNA.

INTRODUCTION

Studies involving the effects of UV irradiation on DNA replication in mammalian cells have shown that CHO cells, which excise only a small percent of the pyrimidine dimers induced, replicate most of their genome after 5 or 10 $J \cdot m^{-2}$ of UV (1). As the DNA synthesized is larger than interdimer distance, replication must get past dimers in parental DNA (2) and, unlike bacteria (3), do so without exchanging many of the lesions to daughter strands (2).

Various experiments have suggested that DNA replication in normal human cells reacts similarly (4,2). However, interpretations of data are difficult for at least three reasons. First, normal human fibroblasts can excise half of the dimers induced by 10 $J \cdot m^{-2}$ UV during twenty four hours after irradiation (5). Second, after this dose, only half the amount of DNA is made during this period as compared to unirradiated cells. Third, DNA replication is affected to greater extent in UV irradiated excision defective Xeroderma pigmentosum cells (6). These facts suggest a close correlation between excision and DNA replication. Hence, it was possible that DNA synthesis seen in UV-irradiated normal cells only occurred in portions of the genome that had undergone excision-repair to remove dimers prior to replication, or that replicating regions were being preferentially repaired by excision. In order to investigate these possibilities the repair of both the DNA replicated and that remaining unreplicated has been monitored at various times following irradiation.

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MATERIALS AND METHODS

Cell Culture and Radioactive Labeling: Normal human fibroblasts, HSBP were routinely grown in Dulbecco's modified medium plus 10% fetal calf serum at 37° in an atmosphere containing 5% CO_2. Cells grown to a monolayer in 75 cm^2 flasks were subcultured at a ratio of 1:3. ^3H-thymidine (s.a. 3 Ci mmol) at 0.03 µCi/ml or ^{32}P ortho phosphate at 0.5 µCi/ml were added and the cells grown to a monolayer.

Cell Plating UV Irradiation and Density Labelling: Cells were plated at 7.10^5 per 150 mm petri dish and forty hours later UV irradiated. Some ^3H labeled cells were sampled to determine the dimers induced, others were incubated in medium containing Fluorodeoxyuridine (0.25 ug/ml) and bromodeoxyuridine (3 ug/ml). ^{32}P prelabeled cells for repair replication studies were similarly incubated except that ^3H thymidine (s.a. 55 Ci/mmol) at 5 uCi/ml was also included in the medium.

Extraction of DNA, Separation of Replicated and Unreplicated Portions: Cells were lysed with 0.5 M EDTA, 0.5% sarkosyl plus 200 ug/ml Proteinase K (Merck), and left at 37° for 8 hrs prior to phenol extraction. Cesium chloride (1.25 g/ml) was dissolved in each sample and centrifuged in a 40 rotor of a Beckman 5-50 at 30,000 rpm for 40 hours. Gradients were collected as 30 fractions of 0.13 ml, 10 λ of each fraction acid precipitated to determine the heavy/light and light/light peaks, and these portions pooled and dialysed against .01M TrisHCl, .001M EDTA, .04M NaCl pH 8.0.

Estimation of Pyrimidine Dimers: This was carried out by determining the number of UV endonuclease sensitive sites (7).

Determination of Repair Replication: Unreplicated and replicated DNA from ^{32}P prelabeled cells was obtained as above. The light strand from heavy/light DNA, and both light strands from light/light DNA were further purified by 2 centrifugations in alkaline cesium chloride (8). ^3H activity found banding with light ^{32}P labeled strands was used as a measure of repair.

RESULTS

The Effect of UV on DNA Synthesis: Table 1 gives the amounts of DNA synthesised in cells during 12 or 24 hours after 5 or 10 $J \cdot m^{-2}$. Cells receiving 5 $J \cdot m^{-2}$ by 24 hours have synthesized as much as unirradiated controls, but after 10 $J \cdot m^{-2}$ only half this amount is made during the same period.

TABLE 1
THE PRECENT OF DNA SYNTHESISED IN UV IRRADIATED HUMAN FIBROBLASTS

	Time after irradiation (hours	
UV dose ($J\ m^{-2}$)	12 hrs	24 hrs
0	100	100
5	56 ± 10	85 ± 13
10	25 ± 6	50 ± 6

Incidences of Pyrimidine Dimers and Repair Replication in Replicated and Unreplicated DNA: Table 2 presents the numbers of dimers left in the DNA replicated or remaining unreplicated at 12 and 24 hours after irradiation. Slightly fewer dimers were frequently observed in replicated as opposed to unreplicated strands but differences never exceeded 20%.

TABLE 2
THE INCIDENCE OF PYRIMIDINE DIMERS AND EXTENT OF REPAIR REPLICATION IN DNA REPLICATED (HL), OR REMAINING UNREPLICATED (LL) AFTER UV.[a]

UV Dose ($J \cdot m^{-2}$)	Time (hrs)	Dimers Remaining per 10^8 daltons		$^3H/^{32}P$	
		HL	LL	HL	LL
5	12	8	7	1.4	1.5
10	12	12	14	2.0	1.8
5	24	4	5	1.8	2.1
10	24	9	10	3.0	3.0

[a]Treatments were as indicated in the text and time refers to the length of post UV incubation in BrdUrd containing medium. The number of dimers immediately after UV is $3.0/10^8/J \cdot m^{-2}$. $^3H/^{32}P$ denotes the degree of excision resynthesis associated with light DNA strands.

Hence, regardless of dose or time after UV, dimers are found in both the replicated and unreplicated DNA. Following 10 $J \cdot m^{-2}$ the DNA replicated during 12 hours after irradiation was incubated with or without UV endonuclease. The heavy daughter strand was larger than the interdimer distance observed in the replicated light strand.

Table 2 also gives the amount of repair synthesis in the replicated and unreplicated portions of the DNA that received UV. The data indicate that after a given dose and post

irradiation incubation the extent of repair is similar regardless of whether the DNA has undergone semiconservative replication

DISCUSSION

The effect of UV irradiation on DNA replication in human cells (Table 1) is greater than observed in CHO cells receiving equivalent doses (1), despite the fact that human cells are able to excise more lesions than CHO cells (5). Following UV, E. coli exhibits a two fold increase in the repair synthesis seen in replicated versus unreplicated DNA, this being absent in a uvrB excision less mutant (9). Such a result would be expected if a preferential excision repair of replicating DNA occurred. In human fibroblasts, although pulse chase experiments inferred semi-conservative replication could get past dimers (5), a preferential excision repair of replicating DNA in normal cells has been suggested by experiments with normal and excision defective XP fibroblasts (10). However, the data presented here clearly indicate that replication gets past dimers and that both the incidence of dimers remaining, and the repair replication of those removed, is the same in portions of DNA replicated or left unreplicated after the UV irradiation of normal human fibroblasts.

ACKNOWLEDGEMENTS

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DISTRIBUTION OF UV-INDUCED DNA REPAIR SYNTHESIS IN HUMAN CHROMATIN[1]

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ABSTRACT The effects of UV dose and repair time on the distribution of repair synthesis in chromatin from human diploid fibroblasts was analyzed using staphylococcal nuclease digestion kinetics (Smerdon, *et al.*, 1978, *Biochemistry, in press*). UV doses from 1.5 to 12 J/m^2 had little effect on this distribution (90 min labeling time): On a unit DNA basis, about 2.5 times as much repair synthesis occurred in nuclease sensitive regions as in nuclease resistant regions regardless of dose. In contrast, the distribution was very sensitive to length of time of repair synthesis. Per unit DNA, the ratio of nuclease sensitive repair sites to nuclease resistant repair sites was almost 5 when a 10 min labeling time was used. With increasing labeling times, this ratio decreased, reaching a value of approximately 2.5 at 90 min.

INTRODUCTION

Interest in the effect of chromatin structure on DNA repair in mammalian cells has been growing rapidly (1-4). To date no studies on the distribution of DNA damage in chromatin from cells treated with UV have appeared. However, data from both our laboratory (4) and Cleaver's (3) indicate that after UV more repair synthesis occurs in staphylococcal nuclease sensitive regions of the genome (primarily linker regions) than in nuclease resistant regions (primarily core regions). In the present communication we examine the effects of UV dose and short repair times on this distribution.

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MATERIAL AND METHODS

Confluent, hydroxyurea suppressed, human diploid fibroblasts (IMR-90 cells; ref. 5), which had been pre-labeled with ^{14}C-thymidine (dThd), were irradiated with 254 nm UV (1 W/m^2) and labeled with ^{3}H-dThd during repair synthesis as previously described (4).

Preparation of nuclei and DNA, digestions with staphylococcal nuclease (Worthington; 5-15 U/ml in 10 mM Tris, pH 8, 0.1 mM $CaCl_2$, 0.25 M sucrose) and data analysis were carried out as previously described (4). Definitions are as follows:

$$\sigma = \frac{\text{specific activity of DNA from repaired cells}}{\text{specific activity of DNA from control cells}}$$

f_S = fraction of repair synthesis sites in nuclease sensitive regions

f_R = fraction of repair synthesis sites in nuclease resistant regions

ξ = fraction of total DNA bases in nuclease sensitive regions.

RESULTS

Effect of UV dose. In Figure 1 the effect of UV dose on the amount of repair synthesis, expressed as σ, is shown for two experiments in which cells were allowed to repair for 90

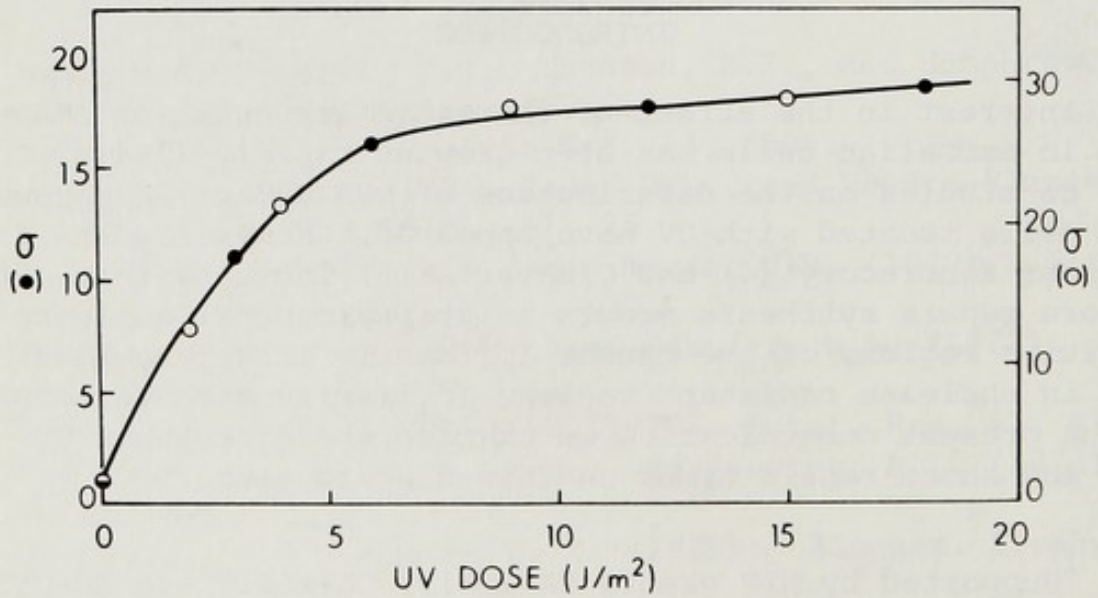

FIGURE 1. Ratio of specific activities (σ) of DNA from cells labeled during repair synthesis and DNA from unirradiated (control) cells as a function of UV dose. Both irradiated and control cells were labeled for 90 min immediately following treatment. Open and closed symbols represent different experiments.

min. The amount of repair synthesis is strongly dependent on UV doses in the range of 0-6 J/m^2 and much less so for doses >6 J/m^2. Indeed, 2 J/m^2 induces almost 50% as much repair synthesis as 12 J/m^2. Also, we note that the value of σ for a given dose varies from experiment to experiment even though the functional dependence of σ on dose remains the same. We have observed that higher values of σ are obtained from cells which are left at confluence for longer periods (T. D. Tlsty and M. W. Lieberman, unpublished results). These data are in agreement with the notion that most of the background in the confluent cell-hydroxyurea system comes from occasional S phase cells in the preparation.

Nuclei and DNA from cells treated with different doses of UV and allowed to repair for 90 min were digested with staphylococcal nuclease, and the data were analyzed as previously described (4). The upper graph in Figure 2 shows the normalized difference plots obtained for 1.5, 4.5, and 12 J/m^2. These curves represent the difference between the digestion data obtained for nuclei and the corresponding purified DNA. The data show that the functional dependence of this difference on the fraction of the genome digested is essentially unaffected by UV dose. The initial and final slopes of these difference curves are proportional to the fraction of repair synthesis sites per unit DNA in nuclease sensitive regions (f_S/ξ) and nuclease resistant regions ($f_R/(1-\xi)$), respectively (4); the intercept of these two slopes yields the fraction of the genome in nuclease sensitive regions (ξ). These parameters were calculated and plotted vs σ for 5 different doses (Figure 2, lower graph). As can be seen, ξ, f_S/ξ, and $f_R/(1-\xi)$ show essentially no change over a 3.5 fold range of σ and an 8 fold range of UV dose. The average values (mean $\pm$ st. dev.) are: $\xi = 0.270 \pm 0.027$; $f_S/\xi = 1.746 \pm 0.110$; and $f_R/(1-\xi) = 0.685 \pm 0.086$. Thus, on a unit DNA basis, the amount of repair synthesis in nuclease sensitive regions is about 2.5 times that of nuclease resistant regions when cells are labeled for 90 min after damage. In addition, estimation of ξ is relatively unaffected by changes in the amount of repair synthesis or UV dose. Also, it is noted that even for low values of σ (in the range of 5-6), representing proportionally less repair synthesis, relatively accurate estimations of ξ, f_S/ξ, and $f_R/(1-\xi)$ can be made with our method.

Effect of repair time. In another series of experiments, we examined the effect of repair time on the distribution of repair synthesis sites in nuclease sensitive and resistant regions. Cells were irradiated with 3 J/m^2 UV and labeled for 10, 20, 30, 60 or 90 min immediately following irradiation. The nuclei and DNA from these cells were digested in the same fashion as above, and normalized difference plots were genera-

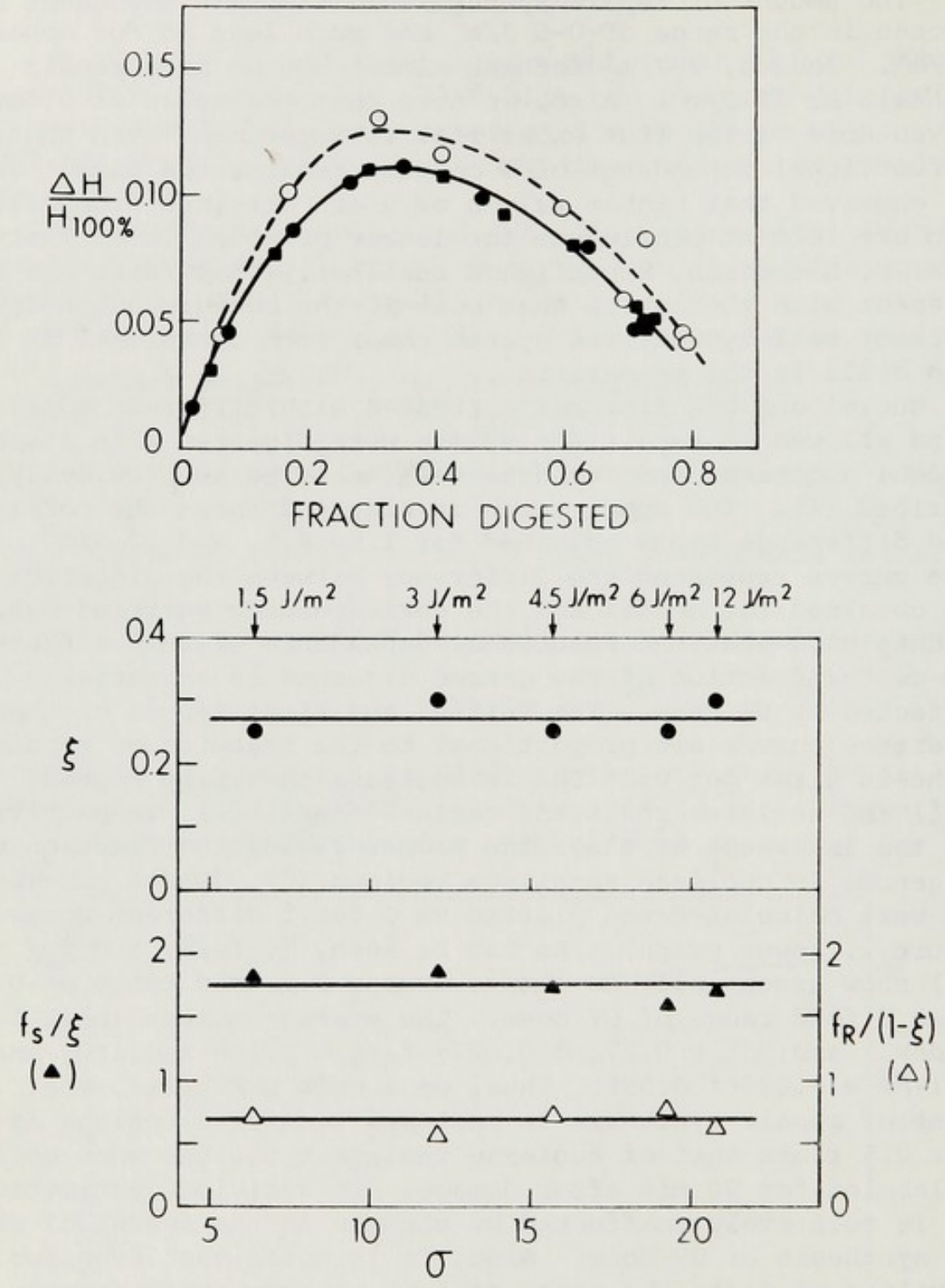

FIGURE 2. Effect of UV dose on staphylococcal nuclease digestion kinetics. Upper graph: Normalized difference plots for 1.5 J/m^2 (o), 4.5 J/m^2 (■) and 12 J/m^2 (●). $\Delta H/H_{100\%}$ represents the difference in acid soluble ^{3}H-CPMs released by staphylococcal nuclease from nuclei and the corresponding purified DNA (ΔH), normalized to the total ^{3}H-CPMs ($H_{100\%}$) (see ref. 4). Lower graph: ξ, f_S/ξ and $f_R/(1-\xi)$ as a function of σ (see Materials and Methods for definitions). The corresponding UV doses are given at the top of the figure (arrows).

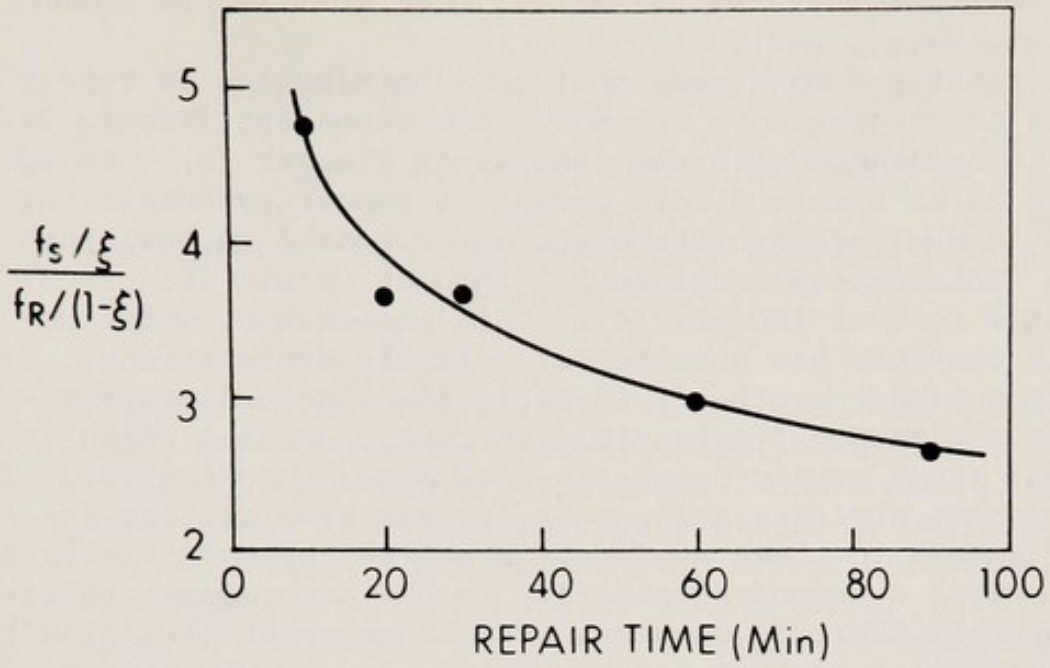

FIGURE 3. Ratio of the amount of repair synthesis per unit DNA in nuclease sensitive (f_S/ξ) and nuclease resistant ($f_R/(1-\xi)$) regions as a function of repair time after 3 J/m^2 UV.

ted. From these, f_S/ξ and $f_R/(1-\xi)$ were calculated and the ratio of these two values was plotted vs repair time (Figure 3). The value of this ratio is almost 5 for the 10 min repair time and rapidly decreases with increasing repair time. Therefore, during the first 10 min after damage, on a unit DNA basis, there is approximately 5 times as much repair synthesis in nuclease sensitive regions of chromatin as in nuclease resistant regions.

DISCUSSION

Previously we showed that linker DNA contained more of the repair synthesis sites than core DNA in cells treated with 12 J/m^2 and allowed to repair for 3 hr (4). In this report we show that the distributions observed at 12 J/m^2 and at lower doses are similar. These results suggest that any perturbations in chromatin caused by UV, at least in the range of 1.5 - 12 J/m^2, do not affect the recognition of repair sites by the nuclease. Furthermore, the distribution of repair synthesis in chromatin is similar for both saturating and nonsaturating conditions (Figure 1 & 2). This finding argues that reduced levels of damage do not enhance the repairability of lesions in nuclease resistant regions relative to that in nuclease sensitive regions. In addition, our studies at low doses (1.5-3 J/m^2) represent conditions for which a number of

authors have demonstrated relatively good survival of human fibroblasts (e.g., 6-8).

Our results demonstrate that the distribution of repair synthesis in chromatin is strongly time dependent (Figure 3) and are in agreement with the results of Cleaver (3). At 10 min there is an almost 5 fold excess of repair synthesis per unit DNA in nuclease sensitive regions compared to resistant regions. This excess drops to 2.5 fold at 90 min (Figure 3) and about 2 fold at 180 min (4). This phenomenon, while undoubtedly complex, has a number of possible explanations. It may be that repair synthesis at early times occurs preferentially in regions most accessible to repair enzymes while at subsequent times repair occurs in less accessible regions. If staphylococcal nuclease and repair enzymes have similar degrees of access to these regions, data like those in Figure 3 would result. A second possibility is that subsequent to repair synthesis there is movement or rearrangement ("sliding") of core particles so that regions which were previously nuclease sensitive become nuclease resistant. Although such movement was undetected in the experiments of Cleaver (3), recently we have found that rearrangements of chromatin structure occur following repair synthesis (9). Such rearrangements probably account for at least some of the apparent change in specificity of repair synthesis for nuclease sensitive regions as a function of time (Figure 3).

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EXCISION REPAIR IN MAMMALIAN CELLS

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ABSTRACT Excision repair after combined treatments of UV and N-acetoxy-2-acetylaminofluorene (AAAF) was studied by three different techniques in cells proficient in UV excision repair and in cells deficient in UV repair. Two patterns of repair were observed: A) in repair proficient cells total repair was additive, and B) in repair deficient cells total repair was much less than additive--usually less than observed for separate treatments--and AAAF inhibited dimer excision. We conclude that in the 1st class of cells pathways for repair of UV and AAAF lesions are not identical, and in the 2nd class the residual excision enzymes are different from those in repair proficient cells.

INTRODUCTION

UV induced pyrimidine dimers and AAAF lesions in DNA are substrates for excision repair in human cells, and AAAF damage mimics UV damage in the following ways: A) both are repaired by a long patch mechanism (~ 100 nucleotides) (1), B) xeroderma pigmentosum (XP) cells deficient in repairing UV damage are also deficient in repairing AAAF damage (1-3), and C) XP cells are more sensitive than normal cells to the cytotoxic and mutagenic activity of both UV and AAAF (4). The amount of excision repair of dimers and AAAF lesions saturates at high doses (5,6). Hence, if AAAF truly mimicked UV damage one would expect that the amount of repair from a combined treatment using saturation doses would be less than the sum of the treatments separately. We investigated cell lines proficient in UV excision repair (normal human, XP variant, ataxia telangiectasia, Fanconi's anemia, and Cockayne syndrome) and repair deficient cell lines (Chinese hamster V-79, and XP groups C, D & E). Three techniques were used to measure repair: unscheduled DNA synthesis measured radioautographically (7), the photolysis of BrdUrd incorporated

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into parental DNA during repair (1), and the loss of sites sensitive to UV endonuclease (8).

RESULTS

The three techniques gave equivalent results (9, 10, and unpublished data) (Fig. 1 and Table 1). We observed the following: 1) repair of UV and AAAF damage saturates, 2) AAAF repair mimics UV repair qualitatively, 3) in repair proficient cells repair is additive at saturating doses of UV and AAAF, and 4) in repair deficient cells, repair due to combined treatment with both agents is much less than additive and actually is inhibited at saturating doses.

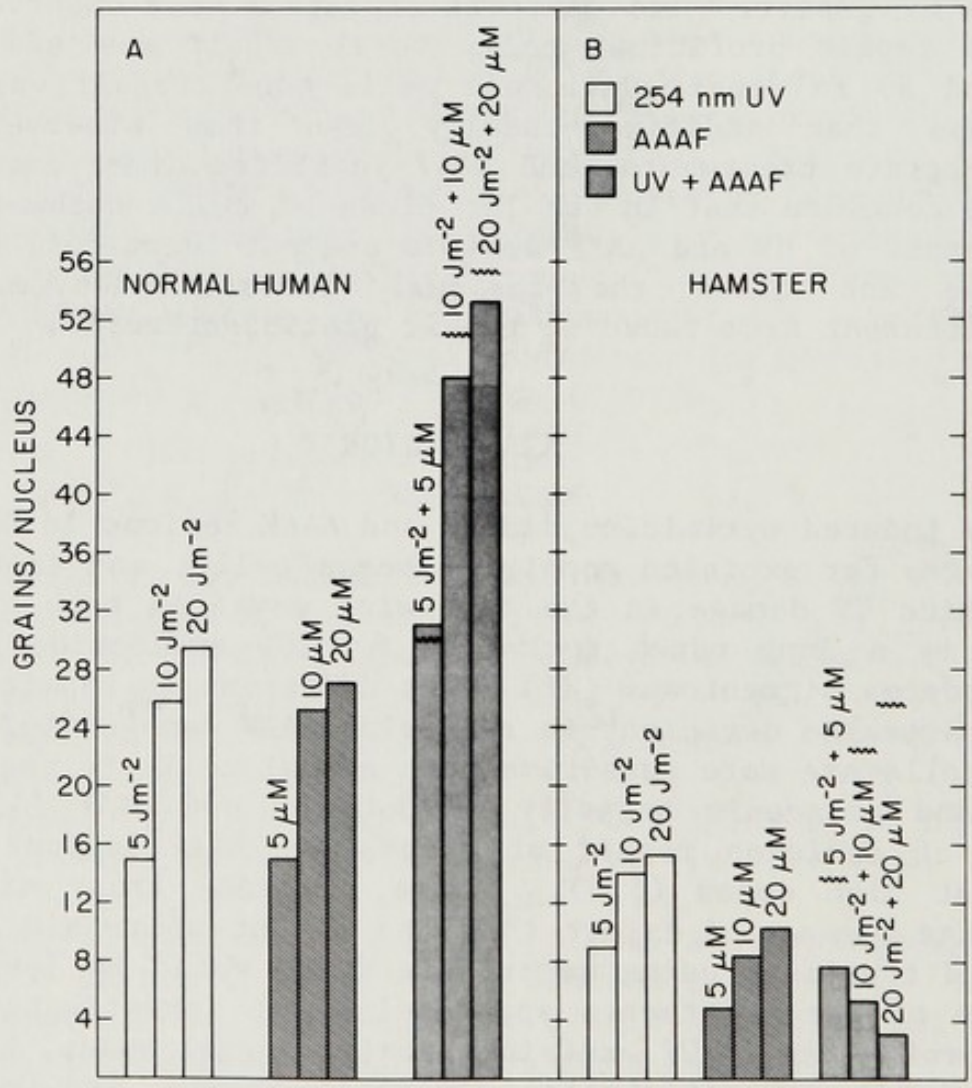

FIGURE 1. The average number of grains per nucleus for various UV doses, AAAF concentrations or combined treatments in normal human and V-79 cells. Wavy line indicates additivity (from references 9 and 10).

TABLE 1

MEASURES OF EXCISION REPAIR IN HUMAN CELLS TREATED WITH UV, AAAF AND COMBINATIONS

Cell line	Unscheduled synthesis[a]			Endonuclease assay[b]		BrdUrd photolysis[c]		
	$20Jm^{-2}$	20μM	$20Jm^{-2}+20\mu M$	$20Jm^{-2}$	$20Jm^{-2}+20\mu M$	$20Jm^{-2}$	10μM	$20Jm^{-2}+10\mu M$
Normal human								
Par Bel (CRL 1191)	18.6	16.1	33	27.5	27.4	3.5	1.4	4.9
Rid Mor (CRL 1220)	17.4	16.4	32.9	23.1	23.3	3.3	1.4	4.4
Ataxia telangiectasia								
NeNo (CRL 1347)	19.7	16.6	35	24.6	24.2	3.2	2.5	5.4
Se Pan (CRL 1343)	22.3	14	33.2	24.5	24.9	3.0	1.4	3.9
AT 4BI	19.6	14.4	35	26.3	26.4	2.2	1.4	3.4
Fanconi's anemia								
Ce Rel (CRL 1196)	15.4	13.9	28.2	27.5	27.4	3.4	0.9	4.4
Cockayne syndrome								
GM 1098	14.3	19.8	33.3	26.1	26.1	3.2	2.6	6.2
GM 1629				26.9	27.0	4.1	2.1	6.6
Xeroderma pigmentosum								
Variant; Wo Mec (CRL 1162)	23.0	17.1	37.2	24.7	24.0	2.8	1.1	4.3
C; Ge Ar (CRL 1161)	3.7	2.2	1.8	3.7	1.4	1.0	0.1	0.1
D; Be Wen (CRL 1160)	6.4	6.6	3.8	3.9	0.8	1.4	0.2	0.4
E; XP2R0 (CRL 1259)	10.8	6.4	2.8	19.3	9.6	4.8	1.0	3.8

[a] Grains/Nucleus incorporated in 3 hr (8 days exposure).
[b] Sites removed in 24 hr/10^8 Daltons.
[c] $\Delta(1/M_n)\times 10^8$ at highest 313 nm dose (12 hr repair).

DISCUSSION

We have no unique explanation for the phenomena observed but suggest the following: since over the range of doses used the damage increases proportionately with dose, the measure of saturation of repair after UV and AAAF is due to saturation of repair systems and not of damage. The rate limiting step in the excision repair process seems to be the incision step (9,10). The following are possibilities to explain why incision is limiting at high doses: A) inaccessibility of lesions to endonucleases (this is ruled out by the fact that at very high UV doses ($\sim 100\ Jm^{-2}$) there is no decrease in the numbers of dimers removed (unpublished data)), B) limiting amount of enzymes or complexes of enzymes[1], C) limiting cofactors[2], and D) similar enzymes but the production of products that inhibit incision or accessibility. This also is ruled out by additivity in repair proficient cells.

[1] For example, incision activities might be part of a small number of repair enzyme complexes and if a later step in repair--say excision--was not completed a subsequent incision might not take place. In such a situation the rate limiting step would be excision but in most experiments would appear to be incision.

[2] For example, a DNA binding protein necessary for efficient endonuclease reactivity.

Some possibilities to explain the similarity between UV and AAAF repair are: A) same enzymes and cofactors or complexes of them working on both lesions, B) different enzymes or enzyme complexes but same cofactors, C) same enzymes but different cofactors, and D) similar or different excision systems but similar accessibility to endogenous enzymes[1]. We can rule out A because of the additivity observed in repair proficient cells and D does not explain the inhibitory effects observed in repair deficient cells.

Hence, we conclude: A) in repair proficient cells additivity implies that AAAF does not completely mimic UV as far as repar is concerned, and that there are different enzymes or complexes of enzymes and cofactors for incision of the two types of damage, B) inhibition of repair after a combined treatment in repair deficient cells seem due to less or different types of endonucleases and cofactors than in repair proficient cells for both UV and AAAF lesions.

We suggested models to explain these data (9,10). There are complexes of repair enzymes and cofactors specific for UV and AAAF in repair proficient cells, and major or minor products inhibit each other in repair deficient cells.

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[1]This possibility ascribes the defect in XP cells to the inaccessibility of their DNA.

A POSSIBLE ROLE FOR POLY ADP-RIBOSE IN DNA REPAIR[1]

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ABSTRACT The synthesis of poly ADP-ribose by BSC or HeLa cell ghosts is dramatically increased by X-irradiation or endonuclease treatment. In a cell-free system it is stimulated by restriction nuclease-digested plasmid DNA according to the type and number of cuts. Thus it probably occurs at or near cut ends of double-stranded DNA and may well occur there in vivo after X-irradiation.

RESULTS

Poly ADP-ribose Synthesis in Ghost Cell Suspensions. In contrast to systems whose preparation involves substantial nuclear disruption, poly ADP-ribose accumulates for as long as 90 minutes in cells rendered permeable to NAD by gentle lysis (Fig 1). The net amount reflects both synthetic and degradative processes. If synthesis is blocked by nicotinamide or NADase, pre-existing poly ADP-ribose disappears exponentially with a half-life of about 10 minutes.

Stimulation by Nucleases and X-irradiation. Synthesis in HeLa or BSC cell ghosts is transiently stimulated by agents that introduce cuts into the cells' DNA (Fig 1). As Miller reported (1), the initial response with either Bovine Pancreatic DNase I or Micrococcal nuclease is proportional to endonuclease concentration (Fig 2A). Over-digestion of DNA is inhibitory. Thus the duration of the boost in synthesis decreases as the DNase concentration is raised. A "pulse" of DNase (i.e. DNase followed by anti-DNase or EGTA) still gives only a transient response. Endogeneous nucleases must have a similar effect, for pre-incubation of ghosts (at 35°) after lysis increases by up to 5-fold their subsequent capacity to synthesize poly ADP-ribose. The effect is lessened by EGTA.

X-irradiation of cells also increases poly ADP-ribose synthesis. Whole cells were irradiated at 0°, at which temperature there is no polymer synthesis, lysed in the presence of [32P]NAD, and then placed at 35°. In BSC cells

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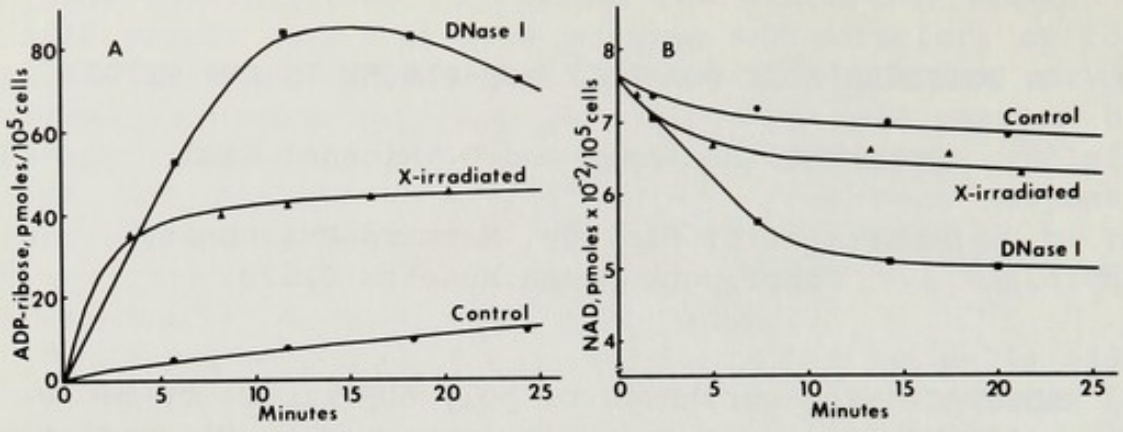

FIGURE 1. A) synthesis of poly ADP-ribose and B) consumption of NAD, in HeLa cells lysed by suspension in 0.05% Triton X-100, 5% dextran T-110, 30 uM [32P]NAD, 10 mM $MgCl_2$, 40 mM tris-HCl, pH 8.0. ●: control, ▲: irradiated with 24 krads at 0° prior to lysis or ■: lysed in the presence of 10 ug/ml DNase I. Incorporation at 35° was terminated by acid precipitation. Surviving NAD was determined by reaction with EF2 (2).

the initial rate of synthesis increases linearly with prior X-ray dose, doubling with 2400 rads (Fig 2B). The boosted response declined at an approximately constant rate if the irradiated cells were incubated at 35° prior to lysis, and approached that of un-irradiated cells in 5-7 minutes, possibly reflecting some repair process.

It is remarkable that the increase after irradiation or a "pulse" of DNase is so short-lived. Nicotinamide, which blocks poly ADP-ribose synthesis extends the period of stimulation. Thus it appears that poly ADP-ribose modifies or conceals the very sites upon which its synthesis depends. Once the available sites are utilized, the rate of polymer synthesis rapidly declines.

Correlation With NAD Breakdown. BSC cell ghosts hydrolyse much NAD by an independent glycohydrolase. However, this activity is weak in HeLa cell ghosts and in these NAD consumption parallels polymer synthesis both during the stimulation by X-rays or nucleases, and during the subsequent return to control levels (Fig 1B). Both activities are inhibited by nicotinamide. Thus the missing NAD must be formed into poly ADP-ribose. However, about twice as much NAD disappears in ghosts of HeLa cells irradiated with 24 krads (5 pmoles NAD/10^5 cells/4 minutes) as acid-insoluble poly ADP-ribose survives. The difference must be due in part to polymer turnover and possibly in part to some undetected acid-soluble product.

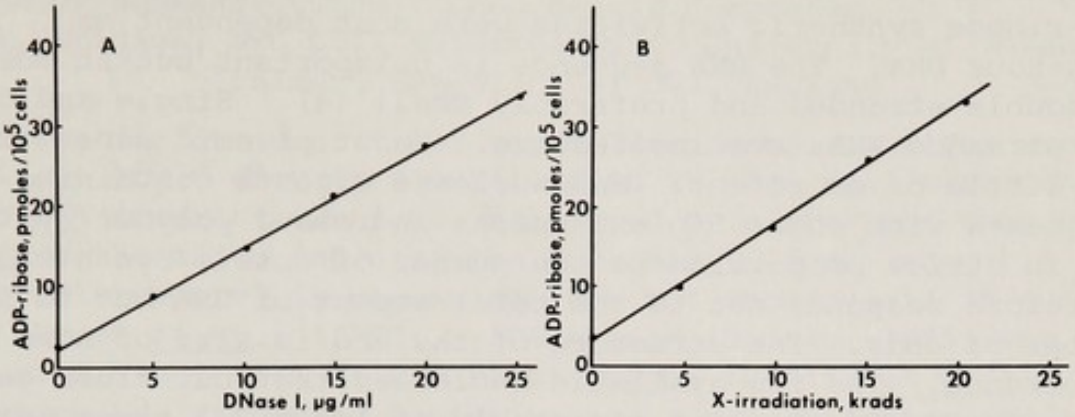

FIGURE 2. Stimulation of poly ADP-ribose synthesis in BSC cell ghosts by A) concurrent DNase I and B) prior X-irradiation. Conditions as in Fig 1. Incubation for 3 min.

Poly ADP-ribose Synthesis by a Cell-free Thymus Extract. These experiments suggest that the synthetic mechanism responds to breaks in DNA. The type of breaks was examined by adding defined fragments of the coli plasmid pBR322 to an enzyme preparation that synthesizes poly ADP-ribose only in response to exogenous DNA. An 0.5 M NaCl extract of calf thymus (3) was passed over DE52 equilibrated in 10 mM

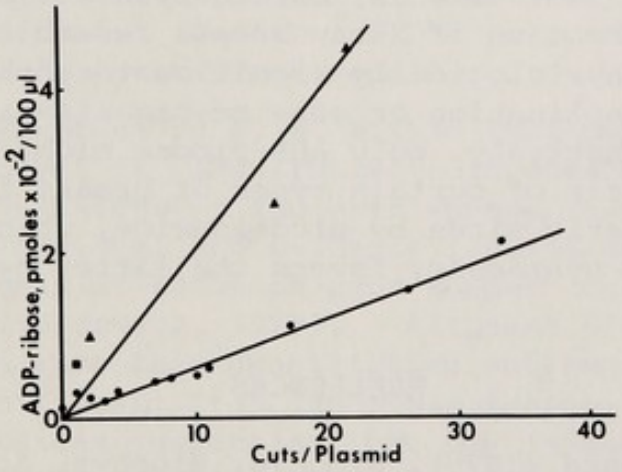

FIGURE 3. Stimulation of poly ADP-ribose synthesis in vitro by digests of pBR322. 200 ul samples, containing 100 ul of thymus extract, 200 uM [32P]NAD, 20 mM tris-HCl, pH 8.0 and 1 ug of DNA were incubated at 25 ° for 4 min. DNA fragments with 5'p extensions of 2-4 nucleotides (number of cuts in parentheses) were given by Eco RI (1), Sal I (1), Bam HI (1), Ava I (1), Taq I (7), Hin f (10) and Hpa II (26). Appropriate combinations were used to give other numbers of cuts. Hinc II (2), Alu I (16) and Hae III (21) gave flush cuts. Pst I (1) leaves a 3'OH extension. ●: 5'p extensions, ▲: flush ends or ■: 3'OH extensions.

phosphate, pH 7.6. Early fractions were pooled whose poly ADP-ribose synthetic activities were most dependent on exogenous DNA. The DNA sequence is unimportant but it must be double stranded and preferably small (4). Single and double stranded RNAs are ineffective. Uncut plasmid supercoils had little or no effect. Endonuclease digests containing fragments with short 5'p extensions increased polymer synthesis in strict proportion to the number of cuts. Synthesis therefore responds not to the total amount of DNA but to the number of ends. The structure of the end is also of some importance. The few available nucleases that cut flush generate fragments that are repeatably at least 2-3 times better than fragments with 5'p extensions. The single available enzyme that leaves 3'OH extensions makes a fragment that may be better still (Fig 3).

DISCUSSION

Since DNA ends of various types are stimulatory *in vitro* it is probable that they act similarly *in vivo* and that a boost in polymer synthesis is a normal consequence of strand breakage caused by X-irradiation. There are two ways in which poly ADP-ribose might be related to repair of the radiation damage. It might be an accidental accompaniment, occuring at the same time as, but independent of, DNA repair. This might be the case if X-ray breaks resembled or were converted to, physiologically significant synthesis sites formed during replication or gene re-organization, for example. Alternatively, poly ADP-ribose might play a primary role in the repair of certain types of breaks in DNA. Preservation of synthetic sites by nicotinamide, which inhibits poly ADP-ribose synthesis, favors the latter hypothesis.

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WORKSHOP SUMMARY: Conditioned Repair Responses

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In a session on conditioned repair responses one might expect that one would discuss situations in which a cell's response to a DNA damaging agent is influenced by a previous exposure. In fact the major topic of the Workshop (SOS or inducible mutagenic repair) is one which does not show itself in such a way (Green et al., 1977). SOS repair (Radman, 1975) was born out of a study of a Weigle reactivation (Defais et al., 1971) in which UV-irradiated bacteriophage survives better and is mutated to a greater extent if plated on bacteria which have themselves previously been irradiated. The SOS hypothesis, that mutagenic repair of DNA damage within the bacterium itself might be mediated by the same inducible repair system as mediates Weigle reactivation, has stimulated a great deal of work since the Squaw Valley meeting in 1974. The first speaker (B. A. Bridges) presented evidence that only mutagenesis in newly-synthesized DNA (e.g. at daughter strand gaps) required protein synthesis consistent with the SOS hypothesis. Mutagenesis at excision gaps or after ionizing radiation (presumed to be at single strand breaks in pre-existing DNA) was chloramphenicol insensitive reflecting the operation of a constitutive pathway. A revertant of a dnaE strain proved to be deficient only in the inducible pathway suggesting the existence of a special function of DNA polymerase III necessary for inducible error-prone repair.

Following a suggestion by E. M. Witkin that perhaps polymerase I might be involved in constitutive mutagenesis, Bridges replied that the nature of the error-prone enzymes was not known for sure but that the evidence implicating polymerase III in mutagenesis at excision gaps seemed convincing (Bridges, Mottershead and Sedgwick, 1976). Although Bridges was inclined to the view that constitutive mutagenic repair was qualitatively different from inducible mutagenesis, he could not exclude Witkin's suggestion that the former might merely reflect a quantitatively lower constitutive level of operation of the latter.

The fact that the dnaE revertant deficient in inducible mutagenic repair showed little extra UV sensitivity raised the question as to whether SOS should be regarded as a repair system or rather as a mutagenic activity. Departing from her published text, E. M. Witkin vigorously defended the repair concept which was based on the fact that mutants which have lost their UV-mutability always show an increase in UV sensitivity. It was pointed out by others that a correlation of increased survival with increased mutagenesis could be clearly

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discerned in Weigle-reactivation and in tif-expressed Uvr^- bacteria. Bridges considered, however, that these were rather artificial situations and there was, moreover, often only a very small increase in sensitivity in UV-immutable bacteria which were excision proficient. Perhaps in Uvr^+ bacteria there was little obvious expression of inducible error-prone repair after UV irradiation because, even though the system was induced, the lesions on which it would normally operate would be removed by the error-free post-replicative excision process postulated by Green et al. (1977) and Clark (these Proceedings).

A related question concerns whether mutations made by the inducible mutagenic system arise at sites of damage (e.g. at some daughter strand gaps) or in undamaged DNA. The latter situation, which Witkin termed untargeted mutagenesis, was indicated by a study on gamma ray mutagenesis in a tif strain (Bridges, 1977). Witkin in discussion suggested that both targeted and untargeted mutagenesis may occur depending upon the amount of replication of damaged compared with undamaged DNA during the period in which induced mutator activity was present. Whereas she felt that targeted mutagenesis occurred in UV-irradiated Uvr^- bacteria she suggested that in Weigle reactivation of phage λ untargeted mutagenesis (seen as mixed rather than pure clones) predominated, citing unpublished work of Radman. A result supporting this was presented by R. Devoret who showed that a very large yield of λ mutants could be obtained from unirradiated phages in a Weigle reactivation experiment if the irradiated bacteria were incubated to allow full induction to occur before infection. Another possibility which was not considered was the induction of mutations in the vicinity of, but not immediately opposite, damaged bases. Such "near targeted" mutagenesis could conceivably occur during repair replication.

The most important gene products involved in the control of UV mutagenesis were the subject of two communications. P. Emmerson described the work from his laboratory showing that the product of the recA gene is the protein known as X (Goudas and Pardee, 1975) and that the tif gene is a missense mutation of the recA gene. He suggested the protein X might act at two levels, one as a protease involved in cleavage of repressor(s) (possibly when modified by an effector) and two as a DNA binding protein at higher concentration.

Notable work on the recA gene product has also been published by K. McEntee but he devoted his contribution to the lexA gene and its product. He described the construction of λ specialized transducing phages convering the region around lexA (= tsl, spr). Preliminary results suggested that the lexA

gene product might be detected as a band of MW 85,000 by polyacrylamide gel electrophoresis. We may clearly look forward to rapid progress in the elucidation of the lexA function. In building models for recAlexA control we should perhaps not forget a number of other mutations that also appear to block UV mutagenesis, e.g. umuC (Kato and Shinoura, 1977), lexC (Johnson, 1977), and the combination of recB and uvrD (K. C. Smith, these Proceedings).

In phage T4 a protein with at least a superficial resemblance to protein X is the product of gene 32, which is required for replication, recombination and repair of DNA. H. Krisch gave a lucid account of the properties of gene 32 protein which, like recA protein, regulates its own synthesis. It can be induced by UV, however, even when RNA synthesis is blocked suggesting that it is regulated at the level of translation. Krisch hypothesized that it may bind to DNA and protect it against nuclease attack. In view of the rather similar suggestions that have been proposed for the recA protein the lack of comparative discussion about the two proteins may perhaps be attributed to the lateness of the hour.

A final paper by P. Schendel described a genuine conditioned response (termed adaptation) in which chronic pre-treatment of E.coli to low levels of alkylation agents resulted in a great resistance to subsequent challenge with a large acute dose. Both mutagenicity and lethality were involved although the two effects were separable since resistance to the killing effect alone requires polA$^+$. Both effects were lexA and recA$^+$ independent and were clearly distinct from any previously described repair system. Adaptation to low mutagenicity correlates well with the ability of the cells to remove 0-6 methylated guanines from DNA. The possibility of an inducible glycosylase had been considered but Lindahl had not been able to detect one. It would not be surprising if there were other similar "adaptive" phenomena waiting to be discovered, not least in mammalian cells.

In final discussion W. R. Rupp raised the possibility that inducible processes might be triggered by an increase in non-superhelical DNA within the cell. Clark pointed out that, except possibly at low doses, recF blocked Weigle reactivation of S13 phage without affecting the induction of suppressor mutations by UV. This raises the question of whether the repair component of Weigle reactivation is distinct from the mutator component, a possibility not inconsistent with the distinction made above between targeted and untargeted effects.

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THE INVOLVEMENT OF E.COLI DNA POLYMERASE III IN CONSTITUTIVE AND INDUCIBLE MUTAGENIC REPAIR

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Summary: Mutagenic repair in newly-synthesized DNA (e.g. at daughter strand gaps) is sensitive to chloramphenicol, consistent with a requirement for an inducible protein. In pre-existing DNA (e.g. after gamma irradiation or at excision gaps after UV) mutagenic repair is insensitive to chloramphenicol indicating that constitutive levels of gene products are adequate or that a different constitutive pathway is involved. A revertant of a dnaE (polC) strain has been isolated that is unable to perform inducible mutagenic repair but carries out constitutive mutagenic repair nearly normally. There appears to be a function of the Pol III replication complex that is specifically required for inducible mutagenic repair but not for constitutive mutagenic repair or for normal DNA replication.

Ultraviolet and ionizing radiations cause base pair substitution mutations in Escherichia coli almost entirely through a pathway controlled by the recA and lexA genes which is known as mutagenic or error-prone repair. The substrate for this repair appear to be certain types of single-strand gap in DNA (Bridges, 1969; Witkin, 1969). Since the Squaw Valley meeting in 1974 two developments have influenced thinking on the mechanism of mutagenic repair. Foremost has been the evidence in favour of the hypothesis (Radman, 1975) that mutagenic repair is inducible (the "SOS" hypothesis, see for example, Witkin, 1977). The second development has been the demonstration that a function of DNA polymerase III (Pol III) is required for mutagenesis after UV (Bridges, Mottershead and Sedgwick, 1976). This has focussed attention upon the possibility that the error-prone step is mediated by Pol III rather than by any newly-induced polymerase. It was suggested that an (inducible?) cofactor might be required to enable Pol III to polymerise in an error-prone way on a damaged template (Bridges, Mottershead and Sedgwick, 1976). This communication briefly describes recent results which link together these two developments.

Mutagenic repair can be both constitutive and inducible

Most of the evidence in favour of the SOS hypothesis has been concerned with situations in which the mutations have arisen in newly-replicated DNA presumably opposite damaged sites on the template strand. Weigle-reactivation, and UV mutagenesis in excision-deficient strains after UV clearly fall into this category, as does the induction of Trp^+ (ochre

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suppressor) mutations after UV irradiation of excision-proficient strains (see e.g. Green et al., 1977; Eyfjord et al., 1977). We had some evidence, however, that UV mutagenesis at other loci in excision-proficient strains and γ-radiation mutagenesis might not involve an inducible pathway (see discussion in Bridges, 1977, page 158).

Table 1. Effect of incubation of E.coli WP2 in growth medium with and without chloramphenicol for the first 60 min after 30 J/m^2 UV on the loss of photoreversibility of mutations to streptomycin and colicin E2 resistance. All bacteria were incubated with chloramphenicol for 90 min prior to irradiation.

Post-irradiation chloramphenicol	Photo-reversal	Time after UV(min)	Induced mutation frequency	Non-photo-reversible mutations (%)
			Streptomycin res	
No	No	0	$7{\cdot}38 \times 10^{-7}$	-
No	Yes	0	$1{\cdot}01 \times 10^{-7}$	14
No	No	60	$5{\cdot}00 \times 10^{-7}$	-
No	Yes	60	$4{\cdot}95 \times 10^{-7}$	99
Yes	No	60	$4{\cdot}65 \times 10^{-7}$	-
Yes	Yes	60	$4{\cdot}58 \times 10^{-7}$	98
			Colicin E2 res	
No	No	0	$9{\cdot}6 \times 10^{-5}$	-
No	Yes	0	$0{\cdot}68 \times 10^{-5}$	7
No	No	60	$9{\cdot}45 \times 10^{-5}$	-
No	Yes	60	$6{\cdot}45 \times 10^{-5}$	68
Yes	No	60	$9{\cdot}99 \times 10^{-5}$	-
Yes	Yes	60	$5{\cdot}97 \times 10^{-5}$	68

We have used chloramphenicol (CAP) to clarify this situation. CAP prevents fixation of mutations (as determined by loss of photoreversibility) after UV irradiation of excision-deficient bacteria (Sedgwick, 1975), and causes rapid loss of suppressor mutants in excision-proficient bacteria (e.g. Witkin, 1969). CAP present both before and after irradiation does not, in contrast, prevent fixation of UV-induced mutations at other loci (e.g. those leading to colicin E2 resistance or streptomycin resistance) in excision-proficient bacteria (e.g. Table 1), nor does CAP significantly affect the yield of mutations induced by γ-radiation.

The CAP data therefore appear to distinguish clearly between mutagenic repair occurring before DNA replication and that occurring after. The former is unaffected by CAP and is presumably constitutive; the latter is CAP-sensitive and could well be inducible. It is therefore likely that mutations induced by ionizing radiation arise as a result of the

operation of a constitutive pathway. Even after UV-irradiation it is my view that most mutations in wild-type bacteria arise in a similar constitutive way as there is evidence that post-replicative error-prone repair occurs at only a small minority of loci (Green et al., 1977; Eyfjord et al., 1977).

A special function of Pol III is involved in inducible mutagenic repair

Following our demonstration that Pol III is required for UV mutagenesis we decided to isolate some temperature resistants of our dnaE strains hoping to obtain some new mutants showing hypo- or hypermutability after irradiation. A number of strains with interesting phenotypes have been obtained; one in particular will be described. CM792 is a temperature-insensitive revertant of CM731 which is itself a dnaE transductant of E.coli WP2 trp from strain BT1026 dnaE. CM792 is not radiation sensitive and mutability via non-repair mechanisms (e.g. spontaneous or induced by ethyl methanesulphonate) is normal or near normal. Normal mutability to Trp^+ is also observed after γ-irradiation and near normal mutability to streptomycin resistance after UV. UV mutagenesis at suppressor loci is, in contrast, severely depressed and there is no Weigle reactivation of bacteriophage T3.

Thus CM792 appears to be specifically defective in the CAP-sensitive (inducible, SOS) mutagenic pathway. We cannot ascribe the CM792 phenotype to the dnaE gene product itself but it seems reasonable to assume that if it is not due to a change in Pol III it is likely to be due to an alteration of some other component of the Pol III complex. We may thus conclude that there is a specific function of the Pol III complex involved in inducible as distinct from constitutive mutagenic repair.

The mechanisms of mutagenic repair

Radman (1977) has suggested that daughter-strand gaps are kept in existence by the 3'-5' exonuclease activity of a polymerase which repeatedly excises any bases inserted opposite a damaged base in the template strand. He further suggests that inducible repair might operate by inhibiting this exonuclease activity thus permitting further chain elongation. Our data support this hypothesis and specifically implicate Pol III.

In addition to CAP-dependence and the involvement of a special function of the Pol III complex, there is another difference between mutagenic repair at excision gaps and at daughter strand gaps. Whereas the former may occur immediately after formation of the gap, the latter (as judged by loss of photoreversibility) shows a clear delay of 15 to 25 minutes. If

Radman's hypothesis is correct at daughter strand gaps one interpretation would be that there is normally a delay of 15 to 25 minutes before inhibition of the exonuclease function takes place in the replicate fork. In pre-existing DNA, however, our data suggest that Pol III exonuclease activity is either inoperative or is immediately inhibited by constitutive levels of inhibitor.

The author acknowledges discussions with Michael Green and Miroslav Radman and the technical assistance of Rex Mottershead.

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STUDIES OF THE RECA AND LEXA GENES OF ESCHERICHIA COLI K-12[1]

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ABSTRACT The zab53 and lexB30 mutations reduce expression of the recA gene on transducing phages and in the cell. Both tsl and spr mutations suppress lexB30 but not zab53 for UV sensitivity and recA expression. Analysis of several recA mutations on λ transducing phages indicate that mutations which prevent SOS functions prevent induction of the transduced recA gene. A ColE1 recA$^+$ hybrid plasmid causes increased PrecA synthesis in lexA$^+$ and lexA$^-$ strains. The lexA genetic region has been analyzed and transducing phages carrying this gene have been isolated. A protein of 85,000 molecular weight has been identified as the lexA protein. These results are consistent with negative control of recA expression by the lexA product. The recA$^+$ product functions directly in its own regulation and in the processes of repair, mutagenesis and prophage induction.

INTRODUCTION

Regulation of the recA gene of E. coli is important for cell survival. The recA protein (called protein X and here designated PrecA) is induced following DNA damage or arrest of DNA synthesis (1-4). Mutations which alter regulation of this gene also affect the expression of mutagenesis, reactivation and prophage induction functions (SOS functions). A particular mutation in the recA structural gene, tif-1,

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causes derepression of the recA gene and expression of SOS functions at 42°C (1, 5, 6). The lexA⁻ mutation blocks recA induction as well as SOS functions and renders cells sensitive to UV irradiation (7). Mount has isolated UV resistant derivatives of lexA⁻ mutants, called tsl and spr, which show conditional or constitutive production of PrecA in the absence of DNA damage or PrecA function (8, 9). These mutations are closely linked to the original lexA⁻ mutation and probably affect the same gene. In general, UV or tif-1 induction of PrecA is accompanied by expression of SOS functions whereas tsl or spr induction is not (10).

Present genetic and biochemical evidence supports a regulatory model in which the recA gene is normally repressed by the lexA protein (1-3). A basal level of PrecA is sufficient to catalyze homologous recombination. In response to DNA damage or temperature shift of a tif-1 mutant, the PrecA is converted to an activated form, PrecA', capable of inactivating the lexA repressor (as well as the λ repressor) and permitting elevated transcription of the recA gene. The lexA repressor is made thermally labile or partially defective by the tsl and spr mutations, respectively. PrecA is made at high levels in these strains because defective lexA protein fails to fully repress the recA gene (11-13).

Two additional mutations, lexB30 (14) and zab53 (15), are closely linked to recA and prevent expression of SOS functions without abolishing recombination proficiency. Complementation experiments with episomes and transducing phages indicate that these mutations are in the recA gene (16, 17 and unpublished results). I have investigated the effect of these mutations upon recA regulation by examining PrecA synthesis in several mutant strains as well as following mutant transducing phage infection of irradiated cells. Using λplexB30, λpzab53 and other recA transducing phages, I have obtained additional evidence that recA is autoregulated and that lexA negatively controls recA expression.

Finally, transducing phages carrying the lexA gene have been isolated. These phages have been useful for studying the lexA genetic region and for identifying the lexA gene product.

METHODS

Transducing phages containing the zab53, lexB30, recA142, recA66, recA29 or recA63 alleles were isolated from independent secondary site lysogens with λcI857 inserted into the srlA gene of the recA mutant strain (18). The λpzab53, λplexB30, λprecA66, λprecA63 and λprecA142-5 phages complement the recombination deficiency of strain KM5123 (lysA argA srlC5 recA123). The λprecA142 phage restores spontaneous inducibility of prophage Ø80 to recA$^-$ hosts. The λprecA29 phage restores resistance to UV irradiation at 42°C in strain KM5123 (λind$^-$). The methods of phage preparation, protein labeling of phage infected and whole cells, gel electrophoresis and isoelectric focusing have been described (1, 18).

Transducing phages containing the lexA genetic region were isolated from λcI857 or λcI857 nin5 insertions in the malB region. Transduction of malE, malK, ubiA and lexA (tsl, spr) was determined in strains C600 malE16, pop1761 (malK3), AN385 (ubiA), DM959 (tsl2) and DM1187 (spr51) respectively.

RESULTS

Transducing Phage Studies. Following λprecA$^+$ or λptif-1 infection of UV irradiated cells, PrecA is made at high levels (Figure 1). Infection by λpzab53, λplexB30 and λprecA142 produces less than 5% of the λprecA$^+$ level of protein (Figure 1). These mutations block induction of SOS functions following DNA damage and are UV sensitive. A UV-resistant revertant of the recA142 mutation, designated recA142-5, was selected and a λ transducing phage carrying this mutation was isolated. λprecA142-5 induces high levels of PrecA following infection of irradiated cells (Figure 1). Similarly, λprecA29 which carries a cold sensitive recA$^-$ mutation induces only a low level of PrecA after infection. The spontaneous UV-resistant revertant phage, λprecA66, restores significant labeling of PrecA following infection (Figure 1). Pulse chase experiments performed with λprecA29 and λplexB30 indicate that these mutant gene products are not rapidly degraded in the cell but are as stable as the recA$^+$ product. The mutations severely reduce ex-

pression of the recA gene on the transducing phages. Isoelectric focusing of these mutant proteins indicates that changes in the level of recA gene expression are, in some instances, accompanied by a change in the isoelectric point of PrecA. (This has been observed for the recA29, recA66 and tif-1 mutations.)

Reduced expression of the recA gene is observed following infection of lexA$^-$ strain DM844 by λprecA$^+$ (Figure 1). The synthesis of λ specific proteins is unaffected by the lexA$^-$ mutation. The reduction in PrecA synthesis in strain DM844 is similar to that seen with zab53 and lexB30 lesions. This result is consistent with the phenotypes of lexA$^-$, zab53 and lexB30 mutants in vivo.

Although similar in phenotype, the zab53 and lexB30 mutations produce proteins with different isoelectric points. λpzab53 makes a protein with an isoelectric point identical to the tif-1 product and λplexB30 induces a protein indistinguishable from PrecA$^+$ in its isoelectric properties (Data not shown.). This result is not unexpected since the zab53 mutation was isolated as a suppressor of tif-1 induction of λ (15) and the lexB30 allele was selected for failure to induce λ during thymine starvation of a recA$^+$ strain (14). My results indicate that the original tif-1 mutation is present in the zab53 strain.

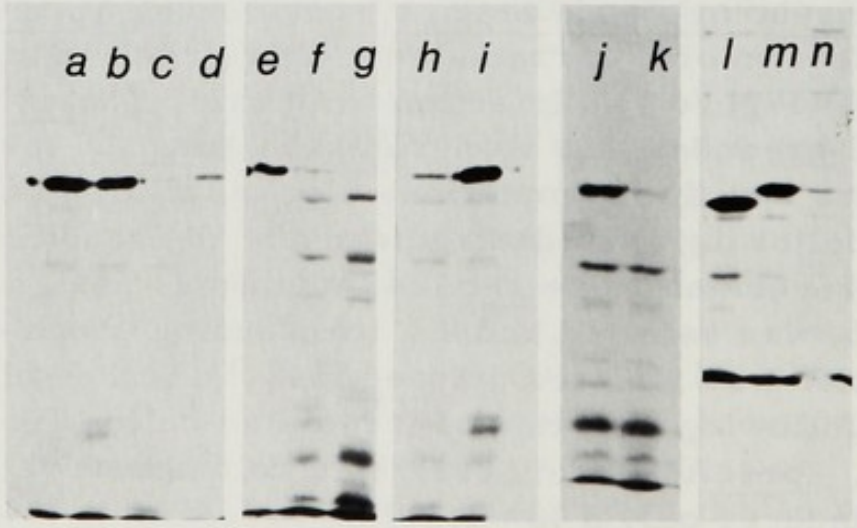

FIGURE 1. PrecA synthesized by mutant transducing phages. a) λprecA$^+$; b) λptif-1; c) λprecA63; d) λprecA29; e) λprecA66; f) λpzab53; g) λprecA142; h) λplexB30; i) λprecA142-5; j) λprecA$^+$; [a-j infections of strain 159] k) λprecA$^+$ infection of lexA$^-$ strain DM844; l) λprecA12; m) λplexB30; n) λpzab53 [l-m infections of tsl strain DM 959].

Another difference between lexB30 and zab53 mutations is shown in Figure 1. The tsl mutation suppresses the defect in PrecA synthesis of the λplexB30 transducing phage but does not suppress the defect in PrecA induction of the λpzab53 variant. This suppression is also observed in vivo with tsl lexB30 (or spr lexB30) and tsl zab53 (or spr zab53) double mutants (Figure 2). The former strains synthesize PrecA at high levels and are UV-resistant. The tsl zab53 mutant strains synthesize low levels of PrecA and are UV-sensitive. I conclude that zab53 and lexB30 block PrecA induction at different steps in the regulatory pathway (see Discussion).

Bacterial Mutant Studies. Strains carrying combinations of recA and lexA mutations were examined for PrecA synthesis by gel electrophoresis of labeled cell proteins. Both tsl and spr mutations allow PrecA synthesis in recA$^+$ and several missense recA$^-$ backgrounds. Although the tsl lexB30 double mutant synthesizes PrecA at a high level and is resistant to UV irradiation (unlike tsl$^+$ lexB30 single mutants), it does not show normal prophage induction at 30°C or 42°C following UV irradiation (data not shown). The recA12 derivatives of tsl or spr strains synthesize a smaller PrecA, whereas an spr recAΔ21 strain (which carries a deletion of the srl-recA region (19)) makes no PrecA. Similarly, the spr zab53 and tsl zab53 strains fail to synthesize PrecA at high levels (Figures 2 and 3).

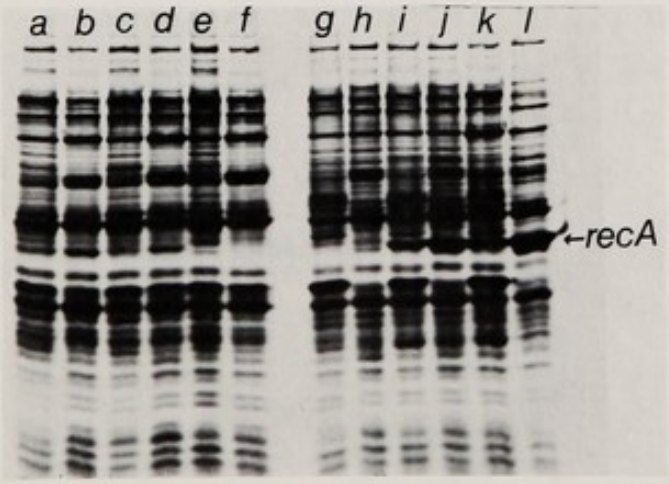

FIGURE 2. PrecA synthesis in tsl and pLC 18-42 strains. a) tsl recA$^+$, 30°; b) tsl recA$^+$, 42°; c) tsl lexB30, 30°; d) tsl lexB30, 42°; e) tsl zab53, 30°; f) tsl zab53, 42°; g) lexA$^-$; h) lexA$^-$ + nal (20 μg/ml); i) lexA$^-$ (pLC 18-42); j) lexA$^-$ (pLC 18-42) + nal; k) lexA$^+$ (pLC 18-42); l) lexA$^+$ (18-42) + nal.

Figure 3 demonstrates the genetic requirements for PrecA derepression. Spr$^+$ derivatives of strain DM1187 show significantly less PrecA synthesis than the parental strain at 30°C. The sfiA$^+$ allele appears to have no effect upon PrecA expression although the spr tif sfiA$^+$ mutant is temperature sensitive for growth on minimal medium due to the tif-1 mutation. A tsl tif sfiA derivative of strain DM1187 synthesizes PrecA at a level comparable to the spr parent. This strain, unlike the tsl tif sfiA$^+$ mutant, is capable of growth at 42°C, indicating that sfiA suppresses tsl induced filamentation. The ability to obtain spr sfiA$^+$ recombinants in these crosses suggests that the spr51 mutation of strain DM1187 does not totally abolish lexA function (see Discussion).

The synthesis of PrecA in strains carrying the ColE1-recA hybrid plasmids (20) is shown in Figure 2. PrecA is made abundantly in strains carrying the pLC 18-42 plasmid. (Similar results were obtained with three different plasmids, pLC 22-40, pLC 21-33 and pLC 24-32.) Addition of nalidixic acid (or introduction of the spr51 mutation), increases the intensity of the labeled PrecA on the gels. However, the uninduced level of PrecA in recA$^+$ lexA$^+$ strains is comparable to the amount of this protein detected in the SOS constitutive strain DM1187. It is not known whether the recA gene on these plasmids is transcribed from its own promoter or from a colicin promoter. Furthermore, it is not known whether both orientations of the recA gene are represented among the four plasmids that were examined.

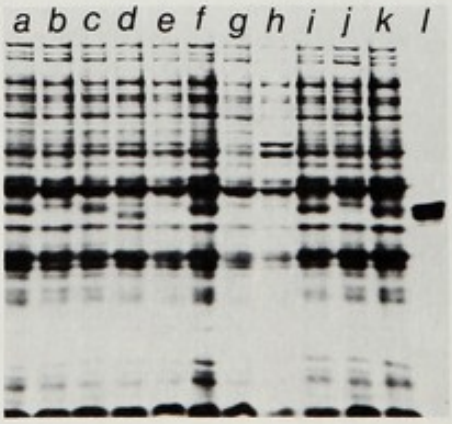

FIGURE 3. PrecA synthesis in strain DM1187 and derivatives. a) spr tif sfiA$^+$; b) DM1187 (λdlexA$^-$); c) tsl tif sfiA; d) spr recA12 sfiA; e) spr zab53 sfiA; f) spr recA6 sfiA; g) spr$^+$ tif sfiA; h) lexA tif sfiA; i) DM1187 (spr tif sfiA); j) lexA tif sfiA; k) spr lexB30 sfiA; l) λprecA.

The pLC 18-42 plasmid suppresses the UV sensitivity of lexA$^-$ strain KM5844 (srlC5 lexA). Survival of the endogenote is 1.5×10^{-4} following a UV dose of 240 ergs/mm^2; survival of the pLC 18-42 derivative at this dose is 0.21. All of the survivors retained the plasmid based upon immunity to colicin and Srl$^+$ phenotype.

PrecA is synthesized at high levels in lexA$^-$ strain KM5844/pLC 18-42 and is further induced by the addition of nalidixic acid (Figure 2). Curing of the plasmid results in the loss of resistance to UV irradiation and the ability to induce PrecA. Expression of SOS functions in strains carrying these plasmids is currently being investigated. Mutants carrying these plasmids have been constructed in which greater than 10% of total cell protein is PrecA. Such strains have been useful in facilitating biochemical studies of this protein.

Transducing Phages for the lexA Genetic Region. The order and linkage of genes in the lexA region is:

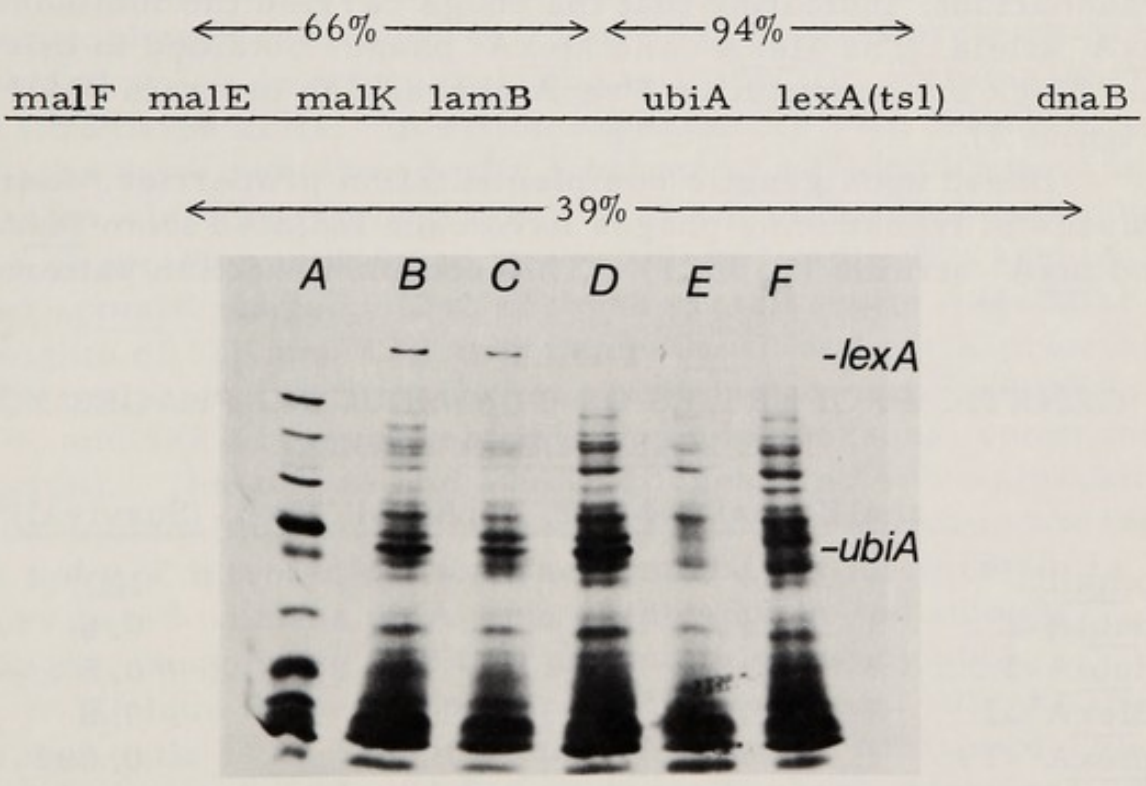

FIGURE 4. Proteins synthesized by transducing phages for the lexA region. a) λ; b) λdlexA$^+$; c) λdlexA$^-$; d) λdubiA-2; e) λdubiA-3; f) λpmalK. The lexA and ubiA proteins are indicated. [Infections b-f in strain 159 (λind$^-$).]

Both tsl and lexA⁻ mutations are cotransduced with ubiA (the octaprenyl transferase gene required for ubiquinone biosynthesis (21)) at frequencies greater than 90%. Transducing phages carrying the ubiA gene were isolated by inserting λcI857 (or λcI857 nin5) into the malB region (22) and inducing these abnormal lysogens by temperature shift. The insertion of λ into the malB cluster occurs in the malF cistron (M. Hofnung, personal communication), and approximately 0.1% of the phages produced transduce ubiA strain AN385 (λ⁺) to ubiA⁺. All of the ubiA phages isolated from three independent secondary site lysogens transduce malE and malK in spot crosses. Approximately 30% of the λubiA phages transduce tsl strain DM959 (λ⁺) to tsl⁺ as judged by growth at 42°C. The large fraction of lexA (tsl) transducing phages among phages selected for ubiA transduction is consistent with the high linkage obtained for these genes in P1kc crosses. λubiA lexA⁻ phages were obtained in an analogous way from Mal⁻ secondary site lysogens of a lexA⁻ mutant. All isolates which transduced tsl (5 were examined) conferred UV sensitivity to the transductant, indicating that the phage carried the dominant lexA⁻ allele. The λlexA⁺ and λlexA⁻ phages obtained in this way block the constitutive PrecA expression in strain DM1187 (Figure 3).

Based upon genetic complementation properties, four classes of transducing phages have been isolated from lexA⁺ and lexA⁻ strains (Table 1). This complementation pattern

TABLE 1
GENETIC PROPERTIES OF TRANSDUCING PHAGES FOR THE MALB-LEXA REGION

	malE	malK	lamB	ubiA	tsl	lexA	(Survival)[a]
λpmalK	+	+	+	−	−	−	ND[b]
λdubiA-2	+	+	+	+	−	−	0.8
λdubiA-3	+	+	+	+	−	−	0.5
λdlexA⁺-1	+	+	+	+	+	+	0.8
λdlexA⁻-19	+	+	+	+	+	+	0.003
λdlexA⁻nin5	+	+	+	+	+	+	0.007

[a]The survival of AN385 lysogen was measured at 100 ergs/mm². The survival of lexA⁻ strain KM5844 is 0.0016 at this dose.

[b]Not determined.

confirms the gene order obtained by P1kc transduction and indicates that these phages contain overlapping substitutions of bacterial DNA which extend from the malB gene cluster. Although characterization of these phages is incomplete at the present time, the λubiA and λlexA phages are defective variants in which the bacterial substitution is in the late gene region of the λ genome. The λmalK phage contains the mal genes in the nonessential b2 region of the chromosome and does not require helper phage for growth.

None of the phages isolated from the malF insertion strains complement ubiC or tyrB mutations. This data suggests that these genes are not located between malF and lexA as reported (23) but are distal to lexA. E. Witkin has observed an extremely low frequency of crossing over in the lexA-dnaB interval (personal communication). I have tested the lexA transducing phages and find that λdlexA$^+$ (but not λdlexA$^-$ or λdubiA) transduces dnaB mutants to dnaB$^+$, indicating that this transducing phage is more extensively substituted.

The proteins synthesized after infection by these transducing phages are shown in Figure 4. Several proteins are labeled which do not correspond to λ specific proteins labeled in irradiated cells. (Infection experiments with defective phages were performed with λ helper at a multiplicity of infection which minimized labeling of λ proteins.) A common set of proteins is induced by the λdlexA$^+$, λdlexA$^-$, λdubiA and λpmalK phages. Two of these proteins with molecular weights of 52K and 37K may be the lamB and malE proteins. The molecular weights of these proteins are approximately 55K and 37K, respectively (M. Hofnung, personal communication). The λdlexA and λdubiA phages synthesize a protein with molecular weight between 30K and 35K which is absent in λpmalK infected cells. The synthesis of this protein is correlated with the ubiA gene of the λdubiA and λdlexA phages, suggesting that this protein is coded by ubiA.

Unique to the gel patterns of λdlexA$^+$ and λdlexA$^-$ infected cells is an 85K protein. No protein of this molecular weight is made after λdubiA or λpmalK phage infections. The identification of this protein as the lexA product is strengthened by two observations: 1) three independently isolated phages which transduce the lexA (tsl, spr) segment (1 lexA$^+$ phage, 2 lexA$^-$ phages) induce the synthesis of this protein

and 2) the close proximity of ubiA and lexA genes (94% linked) makes it unlikely that this large protein is coded by a gene between ubiA and lexA.

In several experiments, two additional proteins have been detected in λdlexA$^+$ infected cell extracts and not in λdlexA$^-$ or λdubiA infections. One of these is a protein of 55-65K molecular weight and may be the dnaB product [The molecular weight of dnaB protein is 55K (24).].

DISCUSSION

Major observations relating to the regulation of the recA gene are the following:

1) Transducing phages carrying recA mutations which block expression of SOS functions fail to induce high levels of PrecA after infection of irradiated cells. Reversion of these mutations to UV resistance results in increased expression of the recA gene. The 'revertant' PrecA may show a shift in isoelectric properties indicating that PrecA functions in the regulation of its own gene.

2) The lexB30 and zab53 mutations reduce expression of recA in lexA$^+$ strains. The lexB30 mutation is suppressed for UV sensitivity and PrecA synthesis by tsl or spr mutations whereas the zab53 lesion is not suppressed by these mutations. The regulatory model for recA predicts that a down promoter mutation in the recA operon would behave like the zab53 mutation - no induction of PrecA following lexA repressor inactivation. Reversion studies and in vitro transcription experiments should define the zab53 lesion more precisely.

3) The ColE1-recA$^+$ hybrid plasmid, pLC 18-42, suppresses the UV sensitivity of lexA$^-$ mutants and restores high levels of PrecA synthesis. These results are most easily interpreted in terms of repressor titration by the multicopy recA operator of the hybrid plasmid. A noninducible (i. e., lexA$^-$) repressor would also be titrated by increasing the gene dosage of the control region. Introduction of an spr mutation in the plasmid strain causes a further increase in PrecA synthesis without inducing treatment.

That lexA may be a repressor of other inducible operons, such as the ColE1 replisome, has been supported by recent experiments of E. Tessman (personal communication). The filamentation of tsl strains at 42°C suggests that lexA

represses the cell division functions defined by sfiA (and presumably sfiB). As with PrecA synthesis, filamentation occurs in tsl strains without PrecA function.

4) An analysis of proteins induced by transducing phages carrying bacterial DNA from the malB-lexA region indicates that the lexA (tsl, spr) product is a protein of 85K molecular weight.

The experiments reported here point to a direct involvement of the recA gene product in reactivation, prophage induction, mutagenesis and other SOS functions. Large amounts of PrecA are necessary for efficient repair of DNA damage. This repair, usually lexA dependent, is independent of lexA when PrecA is made at high levels. The requirement for a lexA$^+$ gene in the expression of SOS functions may be indirect, as a repressor of recA, or the lexA product may participate directly in these processes. Although these data are consistent with a regulatory function for lexA, they do not rule out a duality of function for this protein as observed with the recA product.

ACKNOWLEDGMENTS

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AN *ESCHERICHIA COLI* MUTANT WITH AN ALTERED INDUCIBLE *rec*$^+$/*lex*$^+$ DEPENDENT DNA REPAIR PATHWAY[1]

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85724

ABSTRACT The mutant DM1285, a spontaneous derivative of DM1187 (*lexA spr tif sfi*) (PNAS 74:300-304), makes turbid plaques with λ^+ phage, and clear plaques with λ ind^s phage. DM1285 maps at the *lexA* locus at 90 min. on the *E. coli* K-12 genetic map. After nalidixic acid or mitomycin C, the mutant DM1285 exhibits unusual kinetics of induction of protein X, the *recA* gene product (PNAS 74:5280-5284). At concentrations of mitomycin C (20µg/ml) which induce the *recA* protein in the wild type strain, JM1(*lexA*$^+$*spr*$^+$*sfi*$^+$), only a small amount of *recA* protein is induced in strain DM1285. After nalidixic acid addition (25µg/ml), the *recA* protein is synthesized at an increasing rate with time in JM1 and DM1187; in DM1285, only a burst of *recA* protein synthesis occurs. Two dimensional gel anlysis demonstrated that the pI of the *recA* protein in strain DM1285 was not altered, as compared to its parent, DM1187.

Survival of UV-irradiated phage λ was two - four times higher on unirradiated DM1285 as compared to unirradiated JM1. However, UV-irradiation of DM1285 resulted in no greater than a five-fold increase in survival of the irradiated phage, whereas UV-irradiation of JM1 resulted in a 20-50 fold increase in phage survival.

INTRODUCTION

Exposure of λ lysogenic strains of *E. coli* to an inducing agent such as ultraviolet light (UV) or nalidixic acid leads to phage lytic development. Other bacterial re-

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sponses to DNA damage include filamentation, limited DNA degradation, error-prone DNA repair, and induction of protein X (1).

Both lexA and recA mutants of E. coli are extremely sensitive to ultraviolet light (UV) and a variety of other agents that damage DNA, probably because these mutants are defective in this error-prone DNA repair pathway. Another type of mutant (STS), derived from a lexA tif sfiA parent, expresses error-prone DNA repair pathways constitutively without the cells having received an inducing signal (2). Moreover, phage λ is not able to lysogenize this STS mutant, which carries an additional mutation spr at the lexA locus (2).

The degree of expression of this error-prone DNA repair pathway in strains carrying mutations at either the lexA or recA locus correlates with the amount of induction of protein X, which has now been identified as the recA protein (1,3,4, 5). Both lexA and recA mutants synthesize only a very small amount of the recA protein after DNA synthesis inhibition (1), whereas the STS mutants (with an spr mutation at the lexA locus) synthesizes the recA protein constitutively (1,3). The tsl and tif mutants, which map at lexA and recA, respectively, are temperate-sensitive for the induction of the recA protein (1).

The mutant DM1285 is representative of a class of spontaneous derivatives of the STS mutant DM1187. In DM1285, a mutation at the lexA locus causes an unusual phenotype with respect to the induction of the error-prone DNA repair functions.

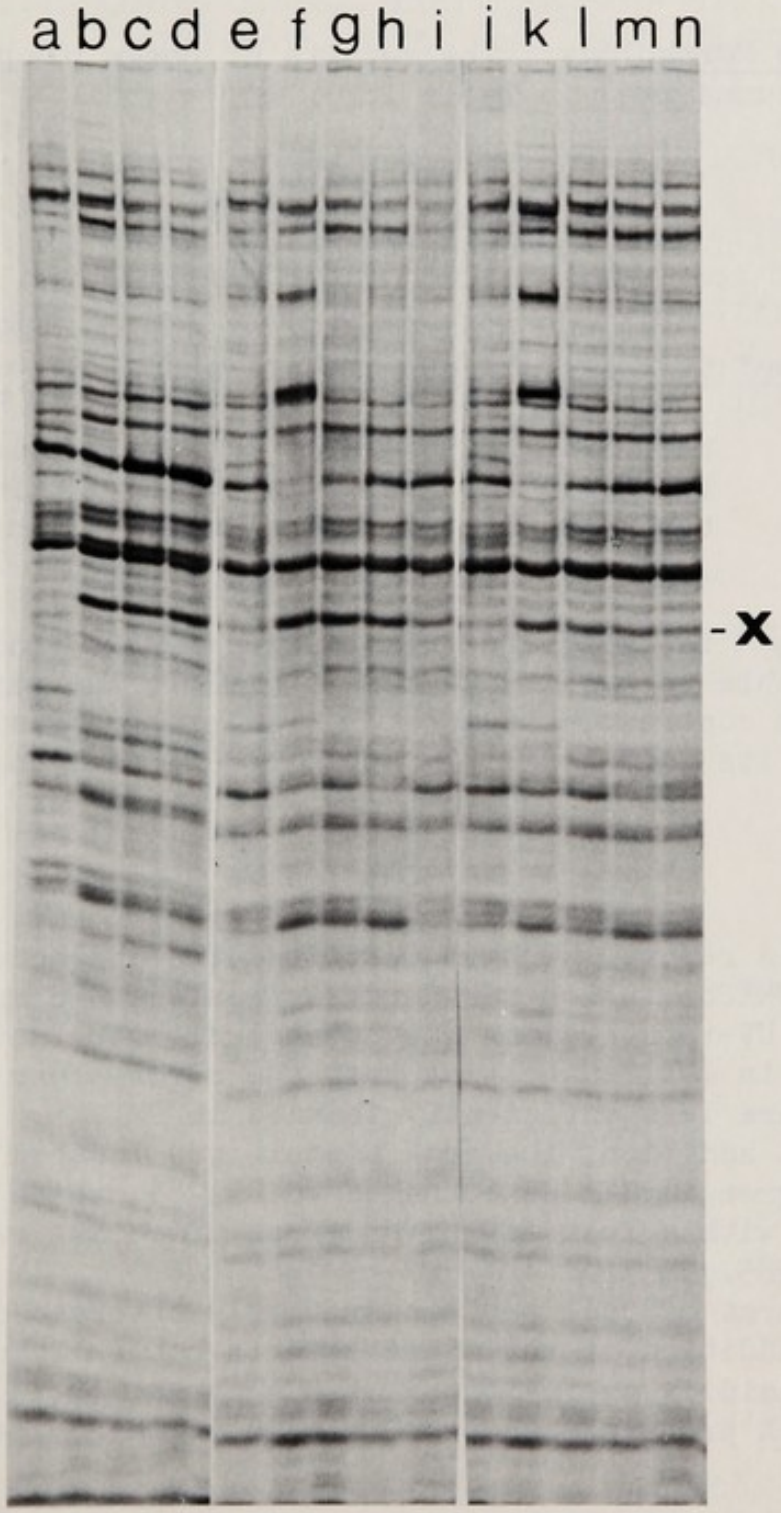
RESULTS

Induction of the recA Protein in DM1285. After addition of nalidixic acid (25-40μg/ml), to strain JM1 ($spr^+tif^+sfi^+$), the recA protein was synthesized at an increasing rate with time, until it reached its maximal rate of synthesis (Figure 1). In contrast, the mutant DM1285 synthesized a burst of protein X after nalidixic acid addition.

The drug mitomycin C can also be used to induce the recA protein. A number of concentrations of mitomycin C were used (15-30μg/ml), but at every concentration, the rate of synthesis of the recA protein in E. coli JM1 was greater than 2 times that observed in DM1285. In both strains JM1 and DM1285, mitomycin C caused the rate of recA protein synthesis to increase with time after addition of the drug, in contrast to the results observed in DM1285 with nalidixic acid.

The isoelectric point (pI) of the recA protein in the mutant DM1285 was identical to that of its parent, the STS mutant DM1187 carrying the spr mutation (3) (data not shown).

Figure 1. E. coli JM1, DM1456, and DM1285 were pulse-labeled with $|S^{35}|$methionine after nalidixic acid addition (30°C), one dimensional SDS polyacrylamide gel electrophoresis was performed, and the dried gel was exposed to Kodak X-ray film (1,3). Slots are a-d: JM1 control; and 10 min., 30 min., and 50 min. after nalidixic acid. e-i: DM1456 control; and 10, 30, 50, and 80 min. after nalidixic acid. j-n: DM1285 control; and 10, 30, 50, and 80 min. after nalidixic acid (DM1456 appears to be similar to DM1285 but has not been fully characterized).



Phage Repair and Mutagenesis in Strains JM1 and DM1285. The survival of UV-irradiated phage λ was 2-4 times higher on unirradiated DM1285 than on JM1. However, UV-irradiation of DM1285 resulted in a less than 5-fold increase in survival of irradiated phage, whereas UV-irradiation of JM1 caused a 20 - 50 fold increase in phage survival. Thus, after UV-radiation, the wild type strain JM1 repaired λ more efficiently than DM1285.

The yield of clear plaque mutants of λ^+ from irradiated JM1 was greater than 5-fold higher than the mutant yield from irradiated DM1285 (see ref. 2 for experimental procedures).

Plaque Morphology. The results of phage plaque-type tests are summarized in Table I.

TABLE I
PLAQUE MORPHOLOGY

Phage	Bacterial Strain: JM1 (spr$^+$ tif$^+$ sfi$^+$)	DM1285 (spr tif sfi sps)	DM1187 (spr tif sfi)
λ^+	turbid	turbid	clear
λind^s	turbid	clear	clear
λind^-	turbid	turbid	turbid

Phage λ^+ is capable of lysogenizing DM1285 and JM1, but λ^+ is not able to lysogenize strain DM1187, the parent of DM1285. In contrast, phage λind^s can not lysogenize DM1285 and its parent DM1187, but λind^s does lysogenize JM1.

DISCUSSION

In this report, we have described the characteristics of a mutant, DM1285, which was derived from the STS mutant DM1187. In DM1285, UV-reactivation and mutagenesis were several-fold lower than in JM1, suggesting that the error-prone DNA repair functions are less efficiently induced in DM1285. Following mitomycin C addition, the recA protein was induced to a much greater degree in JM1 as compared to DM1285, which is also consistent with a less efficient error-prone DNA repair pathway in DM1285.

The burst of recA protein synthesis observed in DM1285 after the addition of nalidixic acid is not fully understood. Nalidixic acid is an inhibitor of DNA gyrase (6,7), and the unusual recA protein induction pattern in DM1285 may result

from an altered interaction between the recA protein, or other proteins involved in DNA repair, and the gyrase.

ACKNOWLEDGMENTS

We thank S. Edmiston for excellent technical assistance.

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INDUCTION AND REPRESSION OF THE recA GENE OF ESCHERICHIA COLI[1]

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ABSTRACT A model is described which accounts for many of the properties of recA and lexA mutants on the assumption that the lexA$^+$ gene codes for a repressor which binds to the operator of the recA gene. Induction of the recA gene occurs when the recA$^+$ product (protein X) already present in the cell in small amounts, is activated by an effector, possibly dNMP. An increased level of dNMP might result from an idling reaction of the 3' to 5' exonucleolytic (proof-reading) function of DNA polymerase III at a stalled replication fork due to an unexcised pyrimidine dimer or a malfunctioning replication complex. The activated form of the recA$^+$ protein then removes the lexA$^+$-coded repressor, possibly by cleaving it.

INTRODUCTION

Protein X, a protein of molecular weight ∿ 40,000, which is synthesised abundantly in recA$^+$ lexA$^+$ cells following DNA damage or inhibition of DNA synthesis, has been identified as the recA$^+$ gene product (1-3). The dependence of protein X synthesis on functional recA$^+$ and lexA$^+$ genes can be explained by assuming that the lexA$^+$ gene codes for a repressor which binds to the operator of the recA$^+$ gene from whence it can be removed by the co-ordinated interaction of an effector and protein X (1,2). In this paper we review the evidence in favour of a repressor role for the lexA$^+$ gene and consider the possible nature of the effector.

RESULTS AND DISCUSSION

Since lexA mutations are dominant in partial diploids (4) and affect the induction of the distant recA gene, it has been proposed that the lexA$^+$ gene product is a repressor (4) which controls the synthesis of the recA$^+$ gene product (1,2). These dominant lexA mutations would be expected to constitute only one class of lexA mutants, in which the repressors are unable

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to interact with inducers and therefore remain permanently bound to the recA operators (1,2). Another class of lexA mutations would be expected to lead to repressor defects which would prevent their binding to the operators. Such mutants should be constitutive for recA$^+$-protein synthesis. The tsl (5) and spr (6) mutations appear to be in this category.

Figure 1 shows an autoradiogram of total proteins in the strain DM936 tsl-1 lexA3 recA1, grown at 30°, and separated by two-dimensional gel electrophoresis. It can be seen that large amounts of the recA protein (protein X) are synthesised constitutively in this strain in the absence of irradiation or any other inducing treatment. This protein X synthesis takes place despite the lexA and recA mutations, as would be expected if tsl-1 were a mutation in lexA which prevented binding of the repressor to the recA operator. At 42°, even more protein X is synthesised (data not shown), in accordance with the known temperature sensitive properties of tsl mutants.

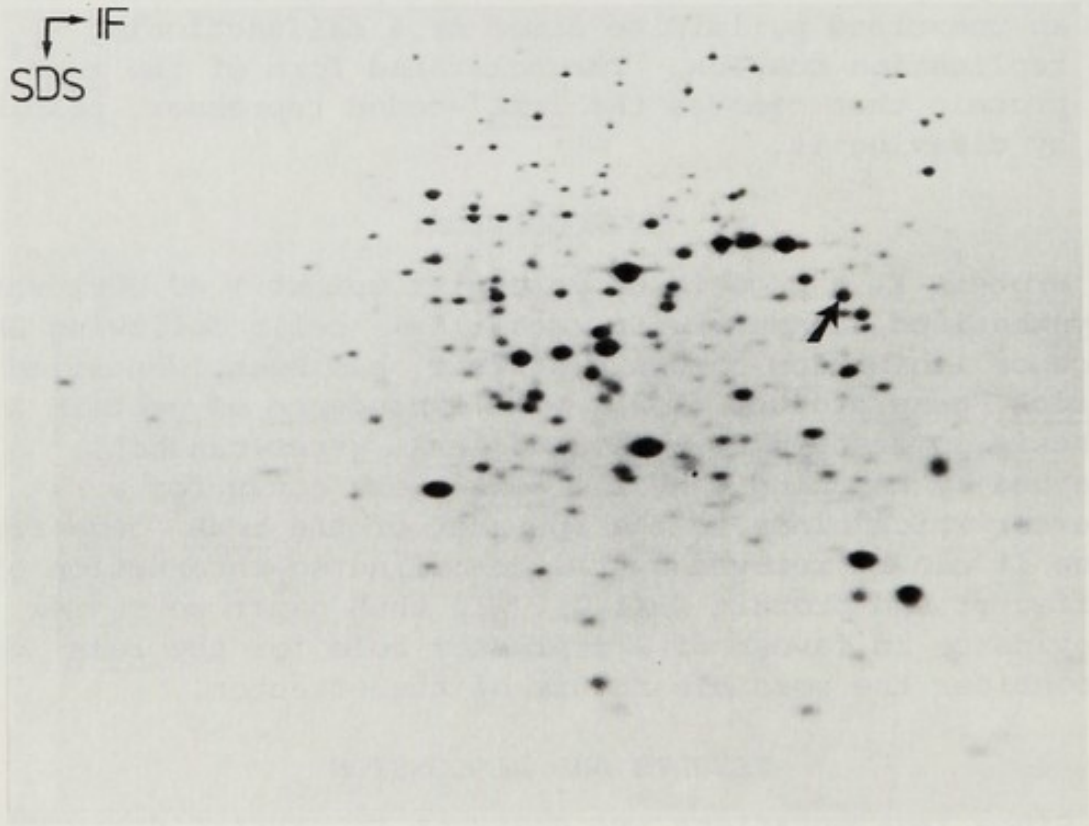

FIGURE 1. Two dimensional gel electrophoresis of total cell proteins of DM936 tsl-1 lexA3 recA1. Cells growing exponentially in low sulphate medium were labelled with 100 μCi/ml ^{35}S for 15 min. Proteins were prepared and separated as previously described (1). The recA protein is indicated.

The spr mutation also leads to extensive constitutive protein X synthesis even in the presence of lexA and recA mutations (2,7). Comparison of the position of protein X in Figure 1 with that in experiments with recA$^+$ cells shows that protein X produced in the recA1 mutant has a more acidic isoelectric point than the wild-type protein. This result provides further evidence that the recA1 mutation is in the gene for protein X and is in agreement with a similar result obtained with the strain DM1415 lexA spr sfiA recA1 (2).

At present the effector is unknown, but it may be produced as the result of post-irradiation degradation or by a stalled replication fork, or both. Gudas and Pardee (8) proposed that the effector was a DNA degradation product resulting from the action of exonuclease V, the recB/C enzyme. This idea was supported by the observation that recB and recC mutants, which can not degrade much damaged DNA (9) did not induce protein X after nalidixic acid treatment. Recently, however, Little and Hanawalt (10) have shown that protein X is induced by UV-irradiation of a recB strain. Thus, degradation by exonuclease V may be unnecessary for induction of X. Nalidixic acid rapidly inhibits DNA replication, possibly by interacting with DNA gyrase (11).

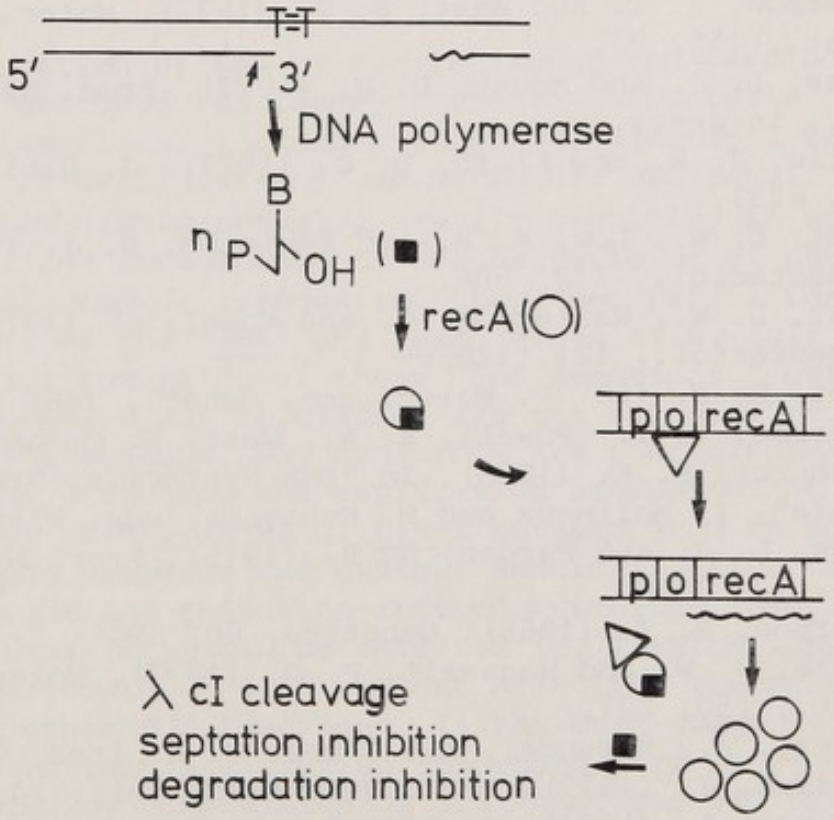

FIGURE 2. Scheme to explain the dependence of synthesis of the recA$^+$ gene product on functional recA$^+$ and lexA$^+$ genes. The lexA$^+$ gene product is shown as a triangle.

The trigger for the induction of protein X could be simply a stalled replication fork such as that caused by nalidixic acid or by the replication complex encountering a pyrimidine dimer in the template strand. An idling reaction could than occur at the 3'OH primer terminus in which DNA polymerase III (or I and II) alternately removes and replaces the 3'-terminal nucleotide (Figure 2). The overall result of this idling reaction of the 3' to 5' proof-reading function of the polymerase (12) would be to degrade the incoming dNTPs to dNMPs, resulting in a rapid increase in the levels of dNMPs. The dNMP could then act as the effector of the *recA*$^+$ protein, altering it in such a way as to permit it to inactivate the *lexA*$^+$-coded repressor.

The build-up of intracellular levels of dNMPs could also inhibit the 3' to 5' exonuclease function of the polymerase by mass action. With its 3' to 5' proof-reading capacity temporarily suspended the polymerase might then be able to use the dimer-containing template, perhaps inserting the wrong bases as it does so, thereby increasing the chances of mutagenesis (for review see ref.13).

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COMPLEXITY IN THE REGULATION OF SOS FUNCTIONS IN BACTERIA[1]

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ABSTRACT DNA damage initiates induction of many functions that promote survival and mutagenesis of bacteria or their phages. In *tif* mutants of *E. coli* these "SOS functions" are thermoinducible. Mutations (Sfi) selected to suppress one Tif-mediated function (filamentous growth at 42°C) may or may not also suppress or alter expression of other SOS functions (prophage induction, error-prone repair). We propose that Sfi mutations participate in SOS regulation via effects on the activity or specificity of cellular protease(s).

INTRODUCTION

Perturbation of DNA by many mutagens and carcinogens triggers a profound regulatory response in bacteria. Numerous inducible functions, including prophage induction, temporary inhibition of cell division and mutagenic DNA repair activity, are expressed simultaneously, usually resulting in enhancement of survival and mutagenesis of the bacteria or of phages which may have infected or lysogenized them (see ref. 1 for review). The products of the *recA*$^+$ and *lexA*$^+$ genes of *E. coli* are necessary for induction of these "SOS functions," but their specific roles in SOS regulation are not yet defined.

In *tif-1* mutants, SOS functions are thermoinducible, although DNA structure and replication are normal at inducing temperatures (2-4). At 42°C, *tif-1* mutants grow in long filaments, induce prophage and exhibit abnormally high frequencies of spontaneous and radiation-induced mutations (3-5). Secondary mutations (Sfi) can be selected (6,7) which suppress filamentous growth at 42°C without affecting thermoinducibility of other SOS functions. It has been suggested (1,6) that Sfi mutations may play an important part in regulating and "fine-tuning" expression of SOS functions via primary

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effects on proteolytic activity. This report describes Sfi mutations that not only suppress filamentous growth of tif bacteria at 42°C, but also alter or eliminate expression of other Tif-mediated thermoinducible functions and decrease the stability of certain abnormal proteins.

RESULTS

In a cross to transfer tif-1 from E. coli K12 to a derivative of B/r (5), Tif expression varied widely in recombinants later found to have received the tif-1 allele (Witkin, unpublished). Recombinants were scored for three thermoinducible SOS functions: filamentous growth, mutator activity and prophage inducibility. A few recombinants expressed all three at 42°C, many only two, others only one, and some showed none of the three and thus appeared Tif^+, yet carried the tif-1 allele. The nonfilamentous recombinants also varied in UV sensitivity at 30°C (Fig. 1a) and in UV mutability (not shown). We suspected that segregation of different Sfi alleles might be partly or even wholly responsible for these variations, but analysis of the "genetic background" differences in the K-B/r hybrids proved difficult. To determine whether Sfi mutations are capable of causing variations in Tif expression such as those observed in the recombinants, we isolated 100 independent Sfi mutants from a fully Tif^- strain, $WP44_s$ (5), by a method described elsewhere (7).

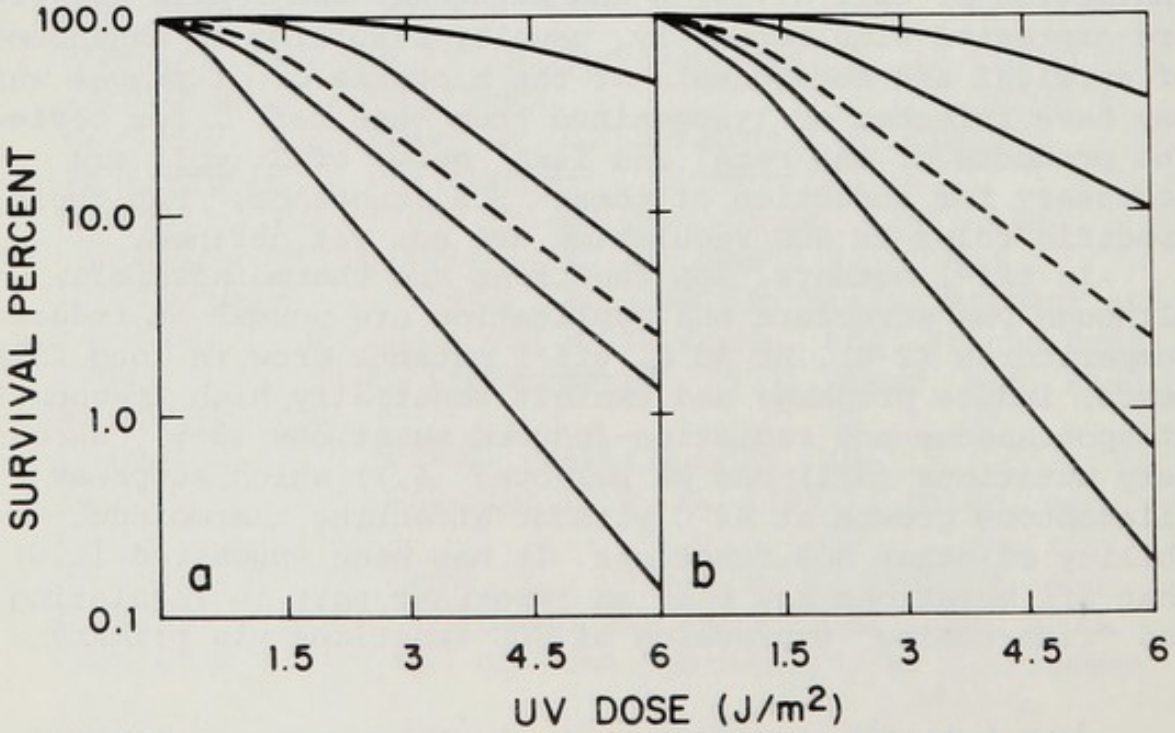

FIGURE 1. Ultraviolet survival curves of tif-1 Sfi recombinants (a) and of tif-1 Sfi mutants (b) at 30°C on 5% SEM agar (5). See text. Broken line, parent strain $WP2_s$.

All of the Sfi mutants were nonfilamentous and viable at 42°C. Their UV sensitivity at 30°C varied (as shown for some of them in Fig. 1b), as did their UV mutability (not shown). Tif expression was as variable among Sfi mutants as among the K-B/r recombinants. Most mutants retained expression of prophage inducibility and mutator activity at 42°C, but the magnitude of the induced mutagenic effect varied widely. At least one Sfi mutant induced prophage at 42°C but showed no thermoinducible mutator activity, and the reverse was true of at least one other. Several Sfi mutants showed none of the three SOS functions at 42°C, yet could donate the tif-1 allele by P1 transduction to recipients capable of expressing the Tif^- phenotype. Translational suppression was not responsible for the Tif^+ phenotype of these mutants. Mapping of Sfi mutations is in progress.

One of us (P. Kirschmeier) has found that Sfi mutations almost invariably increase the rates of degradation of some abnormal proteins (e.g., puromycyl fragments) compared to the filamentous parent strain. At least one Sfi mutant degrades puromycyl fragments but not canavanine-containing proteins more rapidly than the filamentous parent. We have also confirmed Goldberg's observation (cited in ref. 8) that strain B/r degrades puromycyl fragments much more rapidly than its parent strain B. Wild type strain B owes its filamentous growth after SOS-inducing treatments to a lon mutation which is suppressed by a sul mutation (9) in its nonfilamentous derivative, strain B/r (10). Wild type K12, which is lon^+, degrades amber fragments more rapidly than its lon^- mutants (11), which are filamentous (12). Thus, both lon^+ and sul^- alleles suppress filamentous growth (i.e., are in principle Sfi alleles) and also increase proteolytic activity.

DISCUSSION

Sfi mutations, selected for suppression of filamentous growth, may or may not also alter or suppress other SOS functions. Most Sfi mutations markedly increase the rates of degradation of some but not necessarily of all abnormal proteins. Our current working hypothesis is that the primary effect of an Sfi mutation may be to alter the stability of certain proteins selectively (1). In that case, specificity of target proteins unstabilized by individual Sfi mutations could account for the variety of "split" SOS phenotypes described above, as well as for other extraordinarily pleiotropic effects of these mutations (Kirschmeier and Witkin, in preparation).

Our preliminary genetic and biochemical studies indicate that Sfi mutations map at many scattered loci; that they

interact and are therefore difficult to characterize individually, and that different closely related strains frequently carry different (usually cryptic) Sfi alleles.

Proteolytic cleavage of lambda repressor under SOS-inducing conditions has led to the proposal that the recA gene product may have a proteolytic activity (13). Whatever the roles of the recA and lexA gene products in SOS regulation, we are convinced that genes in which Sfi mutations occur are also important elements in control of SOS functions and perhaps more broadly of proteolysis in E. coli.

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tif-1 MEDIATED RECOVERY FROM UV BLOCKAGE OF DNA SYNTHESIS IN *Escherichia coli*: A MECHANISM OF INDUCED MUTAGENESIS?[1]

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ABSTRACT UV doses of less than $3J/m^2$ caused an all-or-none inactivation of DNA replication units in *Escherichia coli* $WP2_s$*uvrA*. This effect required the *recA*$^+$ and *lexA*$^+$ genes and was reversed by expression of the *tif-1* mutation.

INTRODUCTION

The *tif-1* mutant of *Escherichia coli* possesses a thermosensitive ability to replicate DNA past non-coding UV lesions in artificial DNA templates (1). The experiments described below show that the *tif-1* mutants also have a greater ability than *tif*$^+$ cells to synthesise their own DNA after UV-irradiation. To maximise the effect of non-coding lesions in DNA, *uvrA* derivatives of *E. coli* WP2 were used.

RESULTS

E. coli $WP2_s$*uvrA* and $WP44_s$-NF *uvrA tif-1* (2) were grown at 30° in Davis minimal medium (Bacto) supplemented with 20µg per ml of tryptophan. One hour before reaching a density of approx. 5×10^7 cells per ml, half of the culture was transferred to 42°C and 100µg adenine was added per ml. After irradiation with 0, 0.6, 1.2, 1.8, 3.0 and $4.5J/m^2$ of UV, incubation was continued at 30° or 42° for 2 hours. During this period samples were taken at 25 min intervals and labelled with [^{3}H]-thymidine (3). The *amounts* of label incorporated into acid insoluble counts were assayed 5 times in 20 min. The *rates* of incorporation were calculated and are plotted in figures 1 and 2 at the mid-point time of labelling. The rate of DNA synthesis in $WP2_s$*uvrA*, grown at 30°C, increased exponentially reflecting the exponential growth of the

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culture (fig 1a). The initial rates of replication extrapolated to zero time were reduced with one-hit exponential kinetics by UV doses up to 1.8J/m^2 (fig 1a and 1e). The

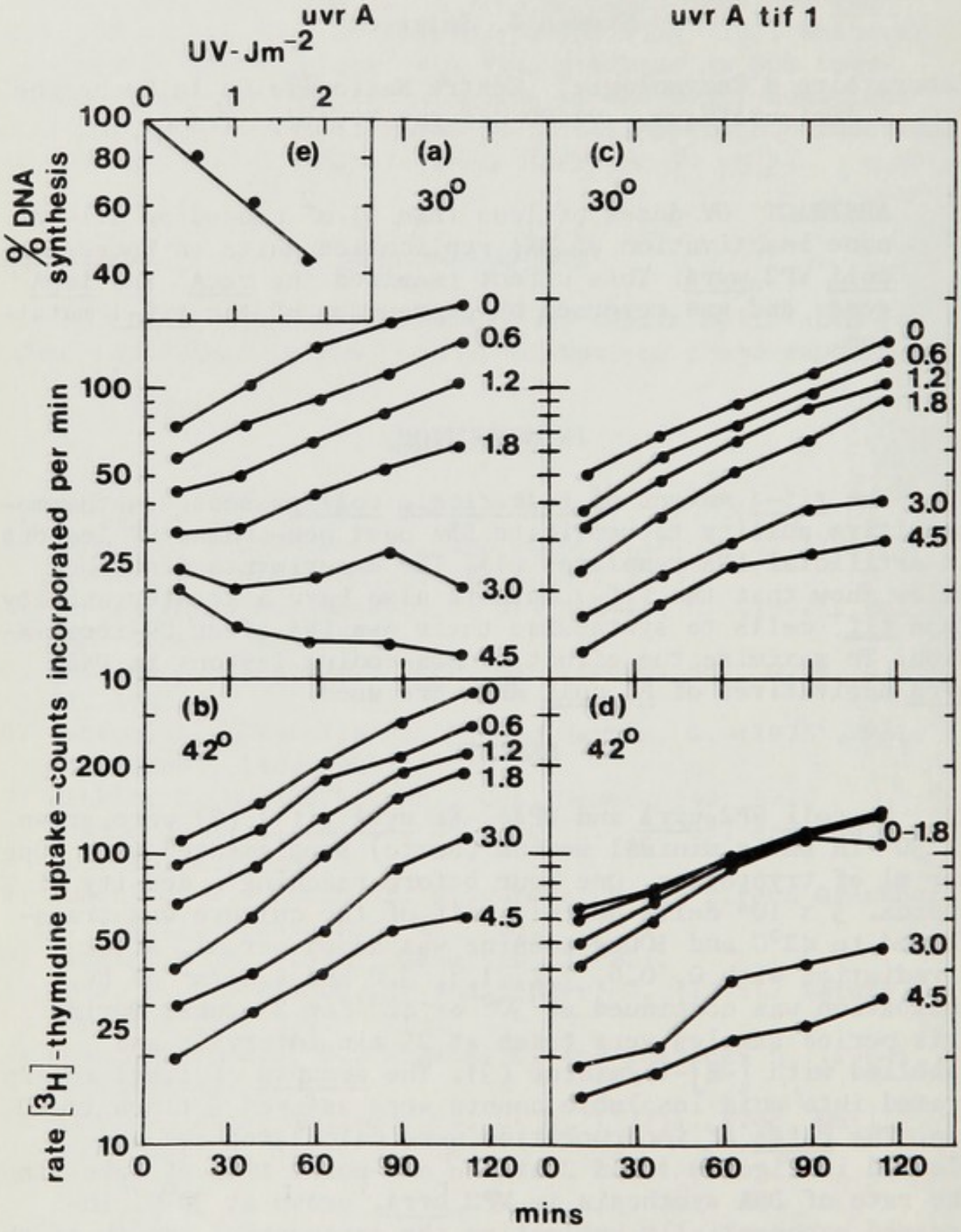

FIGURE 1. Rates of DNA synthesis in WP2$_s$uvrA (a and b) and WP44$_s$-NF uvrA tif-1 (c and d) at 30° (a and c) and 42° (b and d) irradiated with the doses (J/m^2) indicated. 1e. The dose response for UV inhibition of the rate of DNA replication at 30° in WP2$_s$uvrA.

residual rates increased during post-irradiation incubation with the same kinetics as in unirradiated cells. Thus UV irradiation appears to inhibit DNA synthesis by an 'all-or-none' inactivation of replication units. The distinctive "parallel" kinetics for the changes in rate following low doses of irradiation were not seen after 3.0 and 4.5J/m^2 of UV. This indicates an overall slowing of replication in those units remaining active. Similar changes in the rate of DNA synthesis were detected in $WP2_s$ uvrA grown at 42° (fig 1b) and $WP44_s$ uvrA tif-1 grown at 30° (fig 1c) except that synthesis was somewhat more resistant to UV.

When uvrA tif-1 bacteria were grown at 42° in the presence of adenine and irradiated with UV up to 1.8J/m^2 there was little initial decrease in the rate of [^{3}H]-thymidine incorporation. One hour after irradiation in this dose range the rates of DNA synthesis had recovered to the levels seen in unirradiated bacteria (fig 1d). Thus expression of the tif-1 mutation overcomes the blockage of DNA replication seen after UV irradiation of uvrA tif^+ bacteria. Irradiation with 3.0 and 4.5J/m^2 of UV lead to similar changes in the rate of [^{3}H]-thymidine incorporation at 42° in both uvrA tif-1 (fig 1d) and

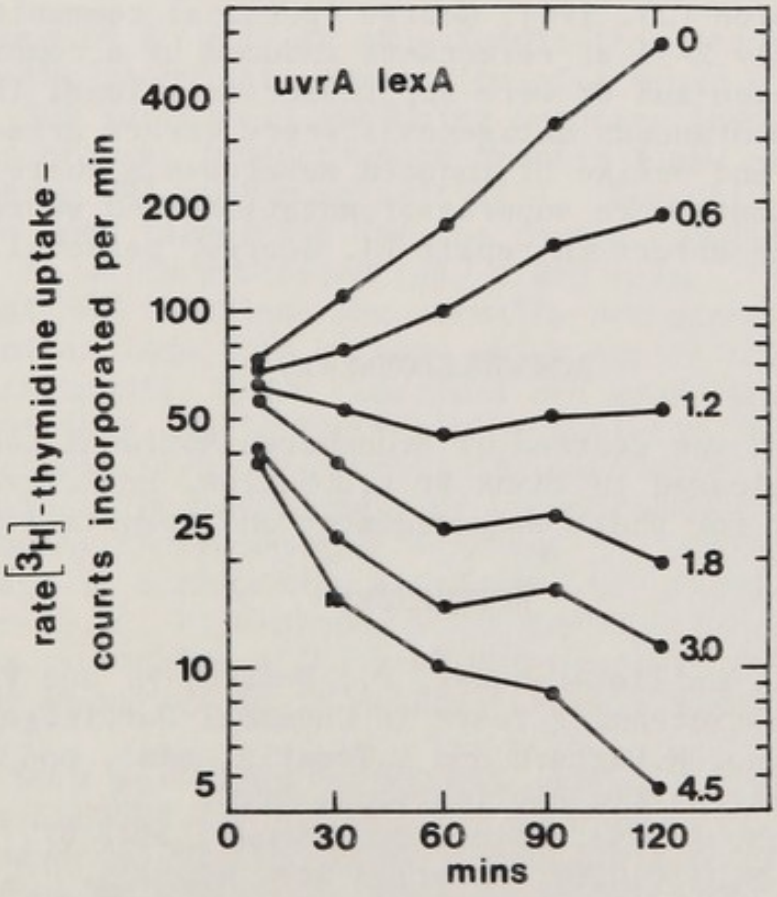

FIGURE 2. Rates of DNA synthesis in CM611 uvrA lexA after UV irradiation with the doses (J/m^2) indicated in the figure.

uvrA tif$^+$ (fig 1b) bacteria.

The apparent all-or-none blockage of replication units was not observed after irradiation of CM611uvrA lexA (fig 2) and WP100uvrA recA (data not shown) grown at 37°C. The initial rates of replication, extrapolated to zero time, showed little reduction by UV irradiation. The effect of UV appeared to cause a gradual slowing of replication.

DISCUSSION

It is concluded that UV doses of less than 3.0J/m^2 cause an all-or-none inactivation of DNA replication units in E.coli WP2$_s$uvrA. This process requires the recA$^+$ and lexA$^+$ genes and is reversed by expression of the tif-1 mutation. It is suggested that UV hypermutability of tif-1 mutants (2,4) stems from their ability to reactivate replication units that are normally permanently blocked because: i)The replication blocks and the mutants induced by tif-1 expression and UV both arose with 1-hit kinetics. UV alone induces mutants with 2-hit kinetics. ii)The blocks to replication and tif-1 enhancement of UV mutagenesis occur (2) after UV doses less than 3.0J/m^2. iii)The blocks to replication persisted at least two hours as did those 'UV lesions' susceptible to the mutagenic effect of tif-1 expression (2). iv)J. George (personal communication) found that only 2-3% of revertants induced by a combination of tif-1 expression and UV were suppressor mutations. This is similar to spontaneous mutagenesis where errors arise in replication, and unlike UV induced mutagenesis where more than 25% of revertants were suppressor mutations and where mutations arise as errors in repair (J. George, personal communication).

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THE ROLE OF PROTEASES IN SOS REGULATION[1]

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ABSTRACT It has been proposed that proteases play a key role in the regulation of SOS functions. We now report that several protease inhibitors of different chemical structures inhibit SOS-dependent inactivation of λ repressor. Additionally, high intracellular concentrations of λ repressor inhibit expression of error-prone DNA repair. This and other evidence for the involvement of proteases in the SOS system are summarized.

INTRODUCTION

Exposure of E. coli to many agents that cause DNA damage results in the coordinate expression of a group of diverse functions (SOS functions) including prophage induction, filamentous growth, production of protein X and expression of an error-prone DNA repair activity (1). Recently, several models have been formulated for the common regulation of SOS functions in which proteases play a key role. These models propose that SOS functions are normally not expressed. Upon formation of an induction signal which may be common to all inducing treatments, these functions are coordinately expressed, possibly by irreversible proteolytic inactivation of their repressors (1-3) or proteolytic modification of existing enzymes (4,5). The involvement of proteases in SOS regulation is supported by three separate findings: 1) SOS induction of λ prophage is accompanied by proteolytic cleavage of λ repressor (3,5), 2) protease inhibitors specifically inhibit the expression of several SOS functions (4-7) and 3) purified preparations of protein X also contain an activity

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which cleaves *in vitro* both λ repressor (J. Roberts, personal communication) and synthetic protease substrates (8).

The only repressor for an SOS function which has been identified and isolated is the λ repressor (9). Roberts *et al.* have extensively studied the SOS-dependent inactivation of this repressor (3,5). They found that UV and mitomycin C induction of λ prophage is accompanied *in vivo* by proteolytic cleavage of λ repressor into 2 fragments (3). This cleavage is dependent on the *lexA*$^+$ and *recA*$^+$ gene products and does not occur in SOS insensitive lysogens (e.g. λ^{ind-} lysogens)(3). They have been able to reproduce this cleavage *in vitro* using extracts from *spr*$^-$ *tif-1 E. coli*, bacteria which express SOS functions constitutively (10). Extracts from cells which are not constitutive for SOS functions fail to cleave λ repressor *in vitro* (5). This *in vitro* cleavage is accompanied by a simultaneous loss of the ability of the repressor to bind to λ DNA (5), leading Roberts *et al.* (5) to suggest that proteolytic cleavage of the λ repressor might be the primary event leading to prophage induction.

These results do not eliminate the possibility that proteolytic cleavage of λ repressor could be a secondary consequence of inactivation of the repressor by another means. However, the inhibition of SOS-mediated λ prophage induction by several protease inhibitors strongly supports the conclusion that proteolytic cleavage of λ repressor is necessary for induction. We initially reported that antipain, a competitive protease inhibitor isolated from *Actinomycetes* (11), inhibited SOS-mediated induction of λ prophage (6). Subsequently, Radman *et al.*(4) reported that TLCK inhibits UV-dependent induction of λ prophage. Using *tif-1 E. coli* lysogenic for λ prophage, we have now screened a variety of inhibitors for their ability to inhibit SOS-dependent inactivation of λ repressor.

RESULTS AND DISCUSSION

The experimental procedure has been published previously (6) and consists of measurement of induction of prophage (by either cell lysis or cell survival) at 42° C in *tif-1* mutants carrying λ$^+$ and λ*cI857* lysogens. At 42° C *E. coli* carrying the *tif-1* mutation express SOS functions without detectable DNA damage or disturbance of DNA synthesis (12). Incubation of *tif-1* bacteria lysogenic for λ$^+$ prophage at 42° C results in the SOS-dependent induction of the prophage (13). *tif-1* bacteria lysogenic for λ*cI857* will also induce prophage at 42° C. However, this induction is not dependent on the SOS system (1). The *cI857* mutation causes the λ repressor to be temperature sensitive. This mutant repressor fails to bind to its operator site at 42° C, resulting in the transcription

of the phage genome, development of progeny and lysis of the cell (1). Every step of the thermal induction of these two prophage is identical except the initial step: inactivation of the repressor itself. Consequently, if a protease inhibitor affects thermal induction of tif-1 (λ^+) lysogens but not tif-1(λcI857) lysogens, it must inhibit SOS-dependent inactivation of λ repressor.

Using this assay, two competitive inhibitors (antipain and elastatinal) and two irreversible protease inhibitors (TLCK and DFP) were found to specifically affect SOS-dependent λ^+ repressor inactivation without affecting SOS-independent λcI857 induction. Two competitive inhibitors (chymostatin and leupeptin), one competitive substrate (TAME) and one irreversible inhibitor (TGCK) did not inhibit SOS-dependent induction without also inhibiting SOS-independent induction of λcI857. The results are summarized below in Table 1.

These results support the suggestion that SOS-dependent induction of λ prophage is dependent on the proteolytic cleavage of λ repressor. Additional evidence suggests that protease(s) might be required for expression of other SOS functions. We have previously demonstrated that antipain inhibits UV mutagenesis in uvrA$^-$ bacteria and thermally induced filamentous growth and mutator activity in tif-1 mutants (6). Additionally, Radman et al. have found that TLCK inhibits UV-induced mutagenesis (4). These findings suggest that proteolytic cleavage of repressors or modification of existing proteins may be necessary for the expression of at least 3 SOS functions. If the expression of SOS functions requires proteolytic modification of either repressor(s) or enzyme(s) and the target molecules possess analogous cleavage sites to that of λ repressor, then λ repressor might act as a competitive inhibitor of the expression of these SOS functions. To test this hypothesis, we introduced a plasmid (pKB252) containing the λrepressor gene (cI)(13), into wp44$_s$NF

TABLE 1
INHIBITION OF SOS-DEPENDENT INACTIVATION OF λ REPRESSOR

Protease Inhibitor	Concentration	Inhibition of SOS Induction
Antipain	0.5 mM	yes
Elastatinal	2.0 mM	yes
Leupeptin	10.0 mM	no
Chymostatin	0.5 mM	no
TAME	2.0 mM	no
TLCK	0.1 mM	yes
TGCK	0.1 mM	no
DFP	0.5 mM	yes

a tif-1 mutant which exhibits an SOS-dependent mutator activity at 42° C. (1) This mutator activity is decreased approx. 50% in strains carrying the pKB252 plasmid (and thus containing large amounts of λ repressor) when compared to wp44$_s$NF and wp44$_s$NF (pmb9). (pmb9 is the parent plasmid of pKB252). This result suggests that λ repressor can inhibit expression of SOS repair and that expression of SOS repair might be dependent on the same protease that is presumed to inactivate λ repressor.

Proteases are known to be involved in control mechanisms in eukaryotes (14). The experiments summarized above suggest that proteases may also play a key role in the regulation of gene expression in prokaryotes. The protease(s) involved have not yet been identified, but recent work suggests that the recA gene might code for a protease. The recA gene product has been identified as protein X (15,16) and Roberts (personal communication) has found that an activity which cleaves λ repressor in vitro copurifies with protein X isolated from spr- tif-1 mutants. An activity against synthetic protease substrates is also associated with these fractions (8). Finally, preliminary work in our laboratory appears to indicate that this activity against synthetic substrates is sensitive to protease inhibitors which inhibit SOS functions in vivo. If the recA gene product can be shown to possess a proteolytic activity, then a biochemical explanation for some of its many pleotrophic effects might be forthcoming.

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TRANSCRIPTIONAL REGULATION OF THE *rec*A REGION OF *E. COLI*

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ABSTRACT We have studied regulation of *rec*A messenger RNA synthesis in *E. coli*. Transcription of the *rec*A region is stimulated at least five-fold by UV-irradiation. Induction of mRNA formation is blocked by mutations in *rec*A and *lex*A. Thus, the *rec*A and *lex*A products are involved in transcriptional modulation of the *rec*A region.

INTRODUCTION

Certain physical and chemical agents (ultraviolet light, mitomycin C, nalidixic acid) inhibit DNA replication in *Escherichia coli*, either by introducing lesions into DNA or by interacting directly with the replication complex (1-3). These same agents elicit a group of cellular responses, the "SOS" or "UV-inducible" functions (4), which include increased mutagenic DNA repair capacity (5), inhibition of cell division (6) and induction of viral prophages such as bacteriophage λ (7). Regulated expression of these inducible functions requires the products of the *rec*A and *lex*A genes (Figure 1) (see ref. 8 for review). Recent experimental findings indicate that the *rec*A protein is itself UV-inducible and may therefore regulate its own expression (9,10).

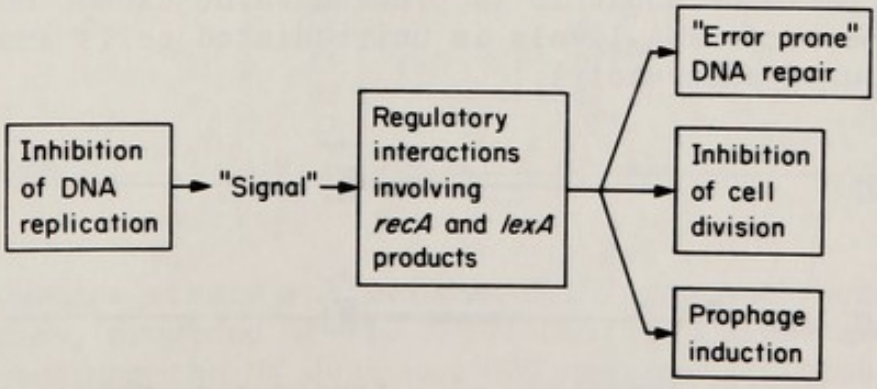

FIGURE 1. Schematic representation of SOS-induction pathway.

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To determine whether transcriptional activation of the *recA* region is a feature of the SOS-induction pathway, we have compared the cellular levels of *recA* messenger RNA before and after UV-irradiation. We find that transcription of the *recA* region is activated by UV-treatment and that the observed stimulation in mRNA synthesis is dependent upon the *recA* and *lexA* products.

RESULTS AND DISCUSSION

Detection of *recA* mRNA. Specific detection of messenger RNA from the *recA* region is facilitated by the use of a λp*recA* transducing phage (11). The genetic structures of two phage we have used to define *recA* mRNA are shown schematically in Figure 2. The λ*srlC* phage carries only the *srlC* gene, whereas λ*srlC recA* bears both *srlC* and *recA* (12). Figure 3 shows the results of an experiment in which a constant amount of DNA from these phage was hybridized with increasing concentrations of RNA from UV-treated cells. Under the conditions employed no specific hybridization to the *srlC* gene was detected (compare λ*srlC* and λ^+). Thus the additional RNA which is complementary to λ*srlC recA* DNA must represent *recA* RNA plus RNA encoded by any genes to the *srl* distal side of *recA*. Since the only λ*srlC recA* specific *E. coli* protein synthesized after phage infection is the *recA* protein (12), all (or nearly all) the RNA found in hybrid with λ*srlC recA* probably corresponds to *recA* mRNA.

UV-induction of *recA* mRNA. The effect of UV-irradiation on transcription of the *recA* region can be seen from the data of Table 1 (lines 1 and 2). In unirradiated cells the level of *recA* mRNA is very low. By 20 minutes after UV-irradiation the fraction of pulse-labeled RNA complementary to λ*srlC recA* is much greater (5-fold) than that hybridizing to λ*srlC*. Thus *recA* transcription is UV-inducible. A comparison of the *recA* specific mRNA from unirradiated and UV-treated cells gives an induction ratio of about 15 (a precise value cannot be given because the *recA* mRNA levels in unirradiated cells are too low to be measured accurately).

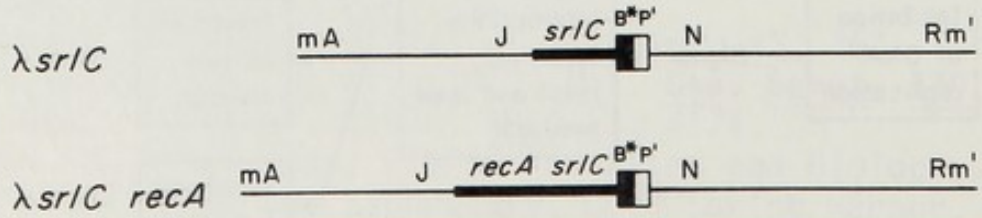

FIGURE 2. Genetic structure of λ transducing phages employed to identify *recA* mRNA.

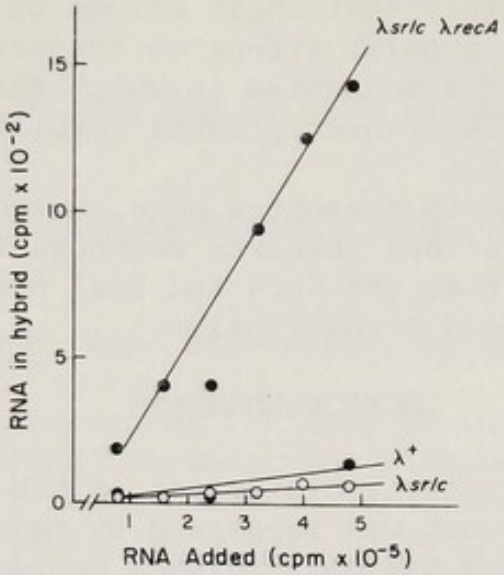

FIGURE 3. Relative hybridization of RNA from UV-treated cells to DNA of λ*srlC recA*, λ*srlC* and λ⁺.

Effects of DNA repair mutations. The effects of several DNA repair mutations on UV-stimulated induction of *recA* transcription are also presented in Table 1. Mutations in *recA* or *lexA* have been shown to prevent induction of SOS-functions (8); lesions in these genes also completely abolish UV-induction of mRNA from the *recA* region. Induction of *recA* mRNA synthesis appears to be less severely affected by *recF*143, a mutation known to affect the SOS-induction pathway under certain conditions (13). Because *recA*1 and *recA*13 are missense mutations

TABLE I

EFFECT OF DNA REPAIR MUTATIONS ON UV-INDUCTION OF *recA* MESSENGER RNA[a]

Percentage of RNA in Hybrid

Genotype	UV	λ*srlCrecA* DNA	λ*srlC* DNA	*recA* mRNA (λ*srlCrecA* - λ*srlC*)
Wild type	-	.10	.07	.03
Wild type	+	.56	.10	.46
*recA*1	+	.06	.03	.03
*recA*13	+	.03	.02	.01
*lexA*3	+	.03	.02	.01
*recF*143	+	.27	.07	.20

[a]The wild type strain was strain AB1157; mutants were isogenic derivatives, provided by Dr. A. J. Clark. Cells were grown in minimal medium; the UV dose was 600 ergs/mm^2. Incubation at 37° was continued for 20 min before pulse labeling. See ref. 14 for RNA isolation and ref. 15 for hybridization procedures.

(9,10), it is unlikely that their effect on UV-induced derepression is due to a polar effect on transcription. Instead, it is suggestive of a mechanism in which the *recA* product itself participates in transcriptional regulation of the *recA* region.

From the experiments presented here, we conclude that UV-induction of SOS-functions involves enhanced transcription of the *recA* gene and that the RecA and LexA proteins participate in regulation of *recA* transcription.

ACKNOWLEDGMENTS

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DOSE RESPONSE RELATIONS FOR UV INDUCED REPAIR PHENOMENA[1]

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ABSTRACT Dose responses for four UV-induced phenomena have been examined for induction with monochromatic UV light at 265nm, using the same irradiation techniques and dosimetry. These show that for W-reactivation of UV-damaged λ-phage, induced inhibition of post radiation DNA degradation and induced radioresistance the relations are similar, though not identical. In the case of the induction of λ-prophage the relation is clearly different, sigmoidal rather than sharply rising at low doses. Inactivation of the λ-repressors evidently involves some additional dose-dependent process.

Introduction. Manifestations of induced repair of DNA include the inductions of λ-prophage, of Weigle (W) reactivation and mutagenesis, and of filamentation, induced radioresistance (irr), induced mutagenic action, induced inhibition of post radiation DNA degradation (prd), and induced respiration halt (1). All of these processes are thought to follow from the derepression of the recA gene, with appearance of relevant cell proteins (2,3). If all processes follow from one such event, then the inductions by ultraviolet light (UV) should be closely related, as coordinate inductions. In strain E.coli AB1157 we conclude that coordinate induction is approximately observed for W-reactivation, induced inhibition of prd, and irr, but not for the induction of λ-prophage.

W-reactivation. To avoid storage changes we prepared λ-phage weekly, by 60J/m^2 254nm UV exposure of AB1157λ cultures growing in Vycor flasks. After two hours subsequent incubation, which cleared the cultures, such "inductates" were further irradiated by 3,000J/m^2 exposure at 254nm, in the same flask. Parallel dilution of unirradiated inductate was made to the irradiated plaque survival level, ca 10^{-5}. For test of W-reactivation these phage preparations, irradiated or diluted control, were adsorbed to AB1157 cells in attachment medium (0.01M $MgSO_4$ in 0.01M TRIS-HCl), after these host cells had been grown in maltose, C-minimal salts + casamino acids.

[1] This work was supported by DOE Contract EY-76-S-05-3631.

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Phage attachment was corrected for free phage by titer of some supernatants, after plating complexes at 10,15, or 20 minutes attachment time. E.coli Q1 was the indicator strain.

W-reactivation was tested by survival of the irradiated phage complexed with host cells irradiated at 265nm, in the attachment medium. Cell irradiations were made using a double water-prism monochromator, with absolute dosimetry and intensity averaging. Numerical considerations for W-reactivation were: unirradiated phage titer n_o before dilution, irradiated phage titer n for unirradiated cells after UV dose D to the phage, and irradiated phage titer N after dose d to the host cells. With sensitivity cross sections S and W(d) for the UV-phage on unirradiated and irradiated cells respectively, an appropriate measure of W-reactivation is:

$$\frac{S - W(d)}{S} = \frac{\ln(N/n)}{\ln(n_o/n)}$$

Such expressions of data from three experiments are combined in Figurel. The dose-response increases sharply at low doses to the cells, with a maximum S - W(d) about 0.25.

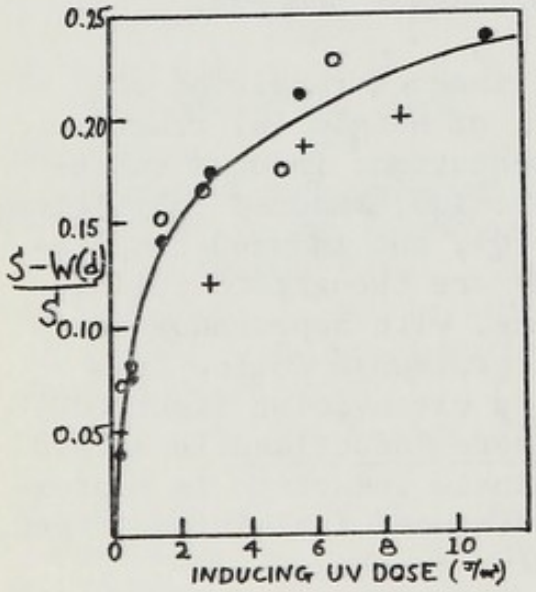

Figure 1. W-reactivation

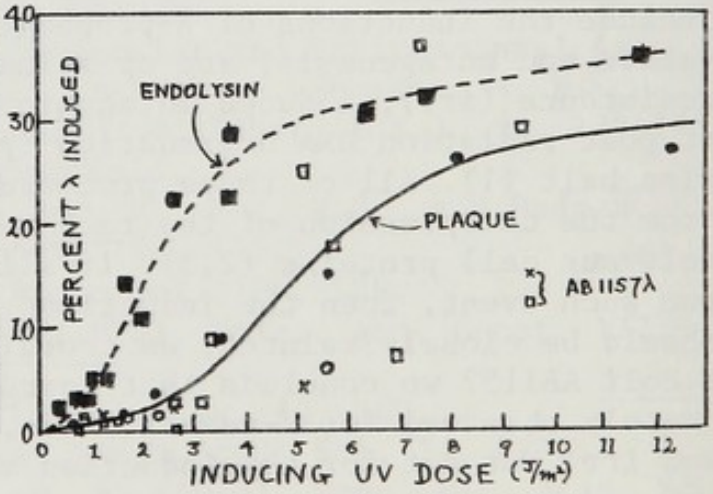

Figure 2. Two methods of observing λ-induction.

The induction of λ-prophage. One of two methods used to study the induction of λ-prophage involved plaque formation. Infectious centers were observed at zero time of incubation and also the yield after burst. For AB1157λ we found the results from the infectious center assay the more variable, though not in conflict with the full burst findings. Strain K12λ, which contains no difference in radiation-involved genes, was more consistent in the infectious center assay. Since it, unlike AB1157 is not lac$^-$, a second way of observing the induction of prophage was possible. As used by Coetzee and Pollard (4), in cells grown on lactose and therefore induced for

β-galactosidase, the cellular enzyme is unable to reach the indicator substrate ONPG while the cell wall is intact. Induction of cell wall endolysin can provide a colorimetric index of the number of cells which have been induced. The results of both methods are shown in Figure 2, mostly for strain K12λ, but in good agreement with observations on AB1157.

The two methods of observation are not in agreement, although both show a sigmoidal response, in which the most rapid increase occurs at $2J/m^2$ for the endolysin assay and $8J/m^2$ for the plaque assay.

Inducible inhibition of post radiation DNA degradation. Cells are labeled with ^{3}H-thymidine, given various inducing doses, incubated for 50 minutes at 37°C, given 50μg/ml rifampin to block further induction and then X-rayed (31krad). Untreated cells show about 75% DNA degradation to acid soluble fragments. After various inducing doses of UV and incubation, cells show less DNA degradation, dependent on dose. The fractions of inhibition estimated from such data are quite similar, except for the dose range, to previous reports for strain WU3610-89. (5). The findings, averaged from three experiments are shown in Figure 3, without data points. The inhibition rises sharply at low doses, with dose-response relationship more like that observed for W-reactivation than for the plaque assay for the induction of K12λ.

Induced radioresistance. Cells are given pre-treatments with 265nm light, incubated for 50 minutes at 37°C, given rifampin and then given a series of X-ray doses to enable a

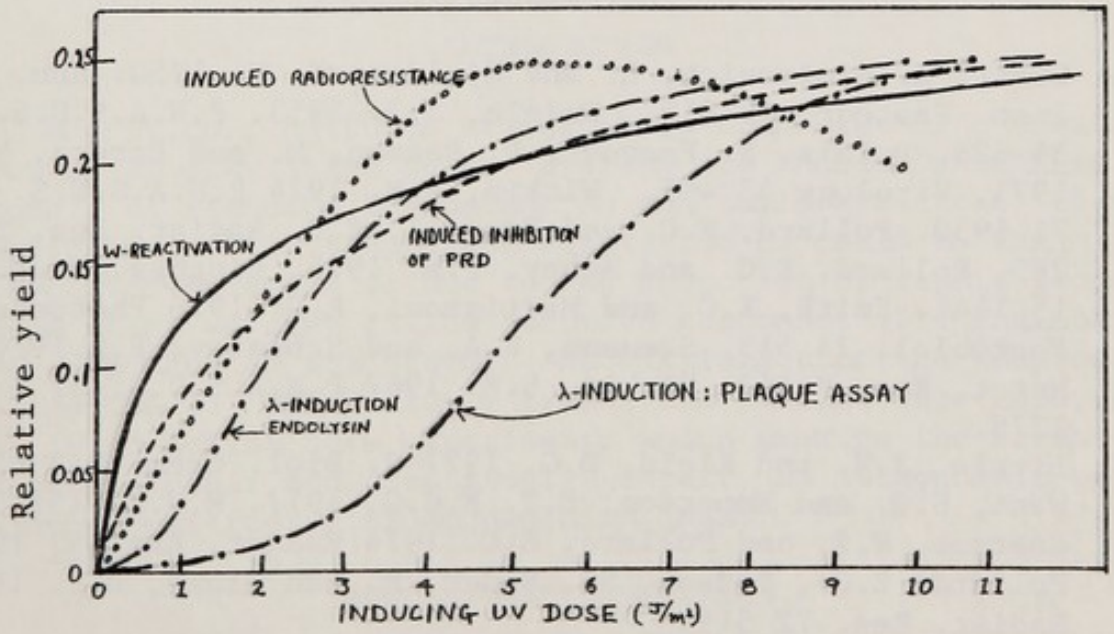

Figure 3. Comparison of the four processes studied. The sigmoidal behavior of λ-induction is in contrast to the initial sharp upward slope of the others.

survival curve to be plotted. Pretreated cells are more X-ray resistant, depending on the UV dose. The amount of resistance so induced was determined by reconstruction experiments, mixing unirradiated and fully induced cells in various proportions. A resulting calibration curve was used to estimate the fraction induced for each UV dose. The findings appear in Figure 3.

Discussion. Figure 3 summarizes the results. Adjustment of all have been made to the 0.25 maximum for W-reactivation. While no pair of induction dose-response curves are closely identical, there is similarity in shape for the curves for W-reactivation, induced inhibition of prd and induced radioresistance. In contrast, the form of the curves for λ-induction is different. The endolysin assay is in rough agreement with the early findings of Marcovich (6) who showed a dose-squared UV induction of λ in his system. Our plaque assay deviates from this relation at the lowest doses.

Previous report on strain WU3610-89 (5) has shown that induced inhibition of prd and UV induced mutagenesis can be brought into coordinate relation if UV light acts in one instance to cause premutational lesions and separately to induce a process converting these to mutations. Induced radioresistance, while not identical, is similar in behavior. The four processes studied here seem to group into 3 which are probably simply related and the fourth, the induction of λ, which is more complex. We feel that the induction of λ is probably also a two-step process in which UV action is needed at least twice.

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PATHWAYS INVOLVED IN REPAIR OF ALKYLATION DAMAGE IN *E.COLI*

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ABSTRACT: Two inducible pathways for the repair of lethal and mutagenic alkylation damage are discussed. These two pathways are at least partially distinct in that the repair of lethal damage requires normal levels of DNA polymerase I, while the repair of mutagenic lesions does not depend on any known repair enzymes. Cells induced for these pathways repair MNNG produced O^6-methyl guanine adducts more rapidly than control cells. The time during which this increased rate of repair occurs correlates well with the period when mutagenesis is reduced, implicating O^6-methyl guanine as an important site of mutation. The adaptive repair pathways reduce the effectiveness of alkylating agents as inducers of SOS functions. As a result we have found conditions where error-prone repair is functional without the coordinate induction of λ prophage or expression of W-reactivation or mutagenesis. Cells also have a constitutive error-free system for the repair of alkylation damage. This system is absent in *lexA* but present in *recA* mutants.

INTRODUCTION

When *E.coli* cultures are grown in a sub-lethal concentration of an alkylating agent, systems are induced which are capable of reducing the mutagenic and toxic effects of these chemicals (1). This apparent adaptation of cells to the effects of mutagens is due to the induction of repair processes which we have called adaptive responses (2). The adaptive responses do not prevent the alkylation of the adapted cell's DNA (3) but actively repair the lesions once incurred (1). We describe here experiments which examine the effect of adaptive repair and constitutive repair on mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

RESULTS AND DISCUSSION

Characterization of the MNNG Adaptive Responses. We have analyzed a variety of different repair deficient mutants for

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their ability to adapt (2). All classes studied have been able to develop resistance to the mutagenic effects of MNNG, but strains mutant at the *polA* locus do not develop resistance to the killing effects of this mutagen. It appears that resistance to killing requires not only an active DNA polymerase I protein but substantial amounts of it, since mutations which cause cells to have reduced levels of functional polymerase also prevent the development of resistance to MNNG killing (4). Since *polA* mutants adapt to the mutagenic effects of alkylating agents, it seems that the two adaptive responses have at least some unshared functional components.

The kinetics of MNNG mutagenesis in unadapted and adapted cultures of strain AB1157 are shown in Fig. 1. Panel A shows that mutagenesis during exposure to 5 µg/ml MNNG is almost totally blocked in adapted cultures for up to 30 minutes. When the challenge dose is increased to 10 µg/ml, the adaptive response can only cope with this flux of mutagen for about 10 minutes, thereafter the resistance to mutagenesis declines rapidly and mutants accumulate. This rapid phase of mutant accumulation is not seen with *lexA* cultures (5) suggesting that it results from the action of error-prone repair enzymes. As the concentration of MNNG is increased above 10 µg/ml, the length of time adaptation can keep mutagenesis at bay decreases by a factor about inversely proportional to the increase in dose.

Expression of SOS Functions in Adapted Cultures. We have shown that adaptation does not depend upon SOS functions nor does the protocol used to adapt cultures lead to the induction of these functions (2). The experiments described in the

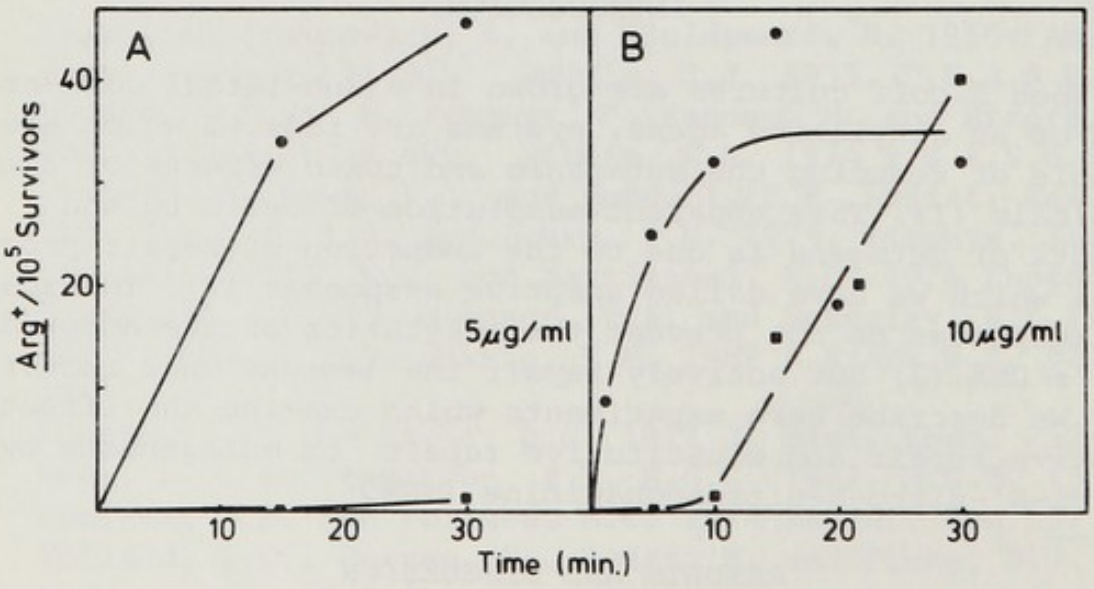

FIGURE 1. Frequency of *Arg*$^+$ revertants observed in samples of an AB1157 culture exposed to 5 µg/ml MNNG (panel A) or 10 µg/ml MNNG (panel B). ●——● unadapted, ■——■ adapted.

TABLE 1
WEIGLE-REACTIVATION AND WEIGLE-MUTAGENESIS IN CELLS CHALLENGED FOR 5 MINUTES WITH VARIOUS AMOUNTS OF MNNG

Culture: experimental parameter	Concentration (µg/ml) of MNNG 0	5	10	20	40	100
Unadapted:						
W-reactivation factor	1	2	3.5	4	5	5
frequency ($x10^4$) of λc mutants	0.9	3	8	-	30	30
Adapted:						
W-reactivation factor	1	-	-	1.2	1.1	1.4
frequency ($x10^4$) of λc mutants	1.4	-	-	0.6	2.7	3.1

last section suggest that error-prone repair is initially blocked by the adaptive system but becomes active if enough alkylation occurs. We thus looked at how the expression of other SOS functions was affected by adaptation. The results of experiments to determine the amount of W-reactivation and W-mutagenesis that could be induced by a challenge of MNNG are shown in Table 1. It can be seen that concentrations of MNNG as low as 10 µg/ml induced unadapted cultures maximally. A 5 minute exposure of adapted cultures to 100 µg/ml MNNG, a challenge sufficient to produce about one Val^r mutant by error-prone repair mediated mutagenesis per 800 survivors, still did not induce W-reactivation or W-mutagenesis to any appreciable extent. The observation of bacterial mutagenesis in the absence of W-mutagenesis is most intriguing. It suggests that the two processes are carried out by different enzymes or by a substantially different intracellular level of the same enzymes.

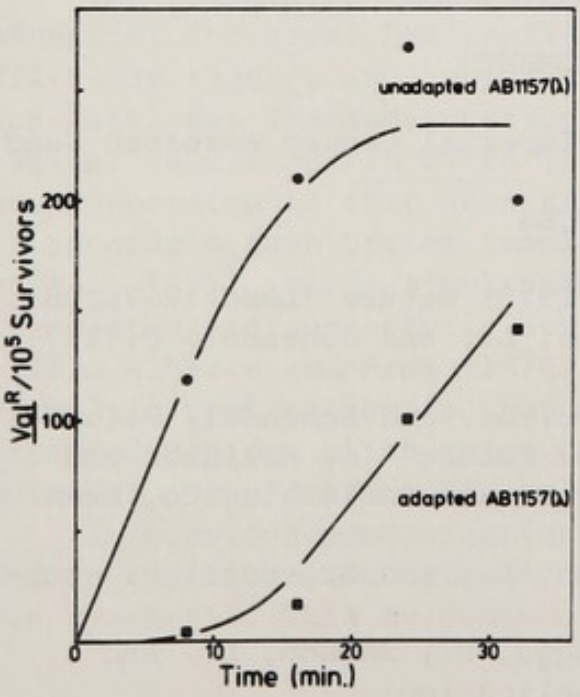

FIGURE 2. Frequency of valine resistant cells after exposure of AB1157 (λ) to 10 µg/ml MNNG.

● ——— ● unadapted culture

■ ——— ■ adapted culture

(The frequency of valine resistant cells per survivor in non-lysogenic *lexA*1 cultures exposed to this level of MNNG for 30 minutes was $120/10^5$ or $15/10^5$ for unadapted and adapted cultures respectively).

Another example of the separate expression of two SOS functions is shown in Fig. 2. Here is shown the kinetics of mutation of unadapted and adapted λ lysogens during exposure to MNNG. As with non-lysogens, error-prone repair mediated mutagenesis begins almost immediately in unadapted cultures and after 10 minutes in adapted cells. Thus individual cells are able to express error-prone repair without inducing λ.

Repair of O^6-methyl Guanine Lesions by Adapted Cells. We have looked at the ability of adapted and unadapted cells to remove lesions put into their DNA by radioactive MNNG. Of the three major purine adducts produced, N^7-methyl guanine is removed in neither adapted nor unadapted cells, N^3-methyl adanine is removed by both at the same rate, but O^6-methyl guanine is removed in adapted cultures at a much higher rate than that found in unadapted cultures. These results strongly implicate the O^6-methyl guanine lesion as one of the major sites of chemically induced mutation.

lex$^+$-dependent Error-free Repair. We have found that at levels of alkylating agent too low to induce error-prone repair, *lexA* cultures were actually more readily mutagenized than their *lex*$^+$ parents. For example, the exposure of a strain carrying the *lexA*1 mutation to 0.5 μg/ml MNNG for 5 minutes leads to the production of 8-10 times as many mutants as a similar challenge to strains with *lexA*$^+$ *recA*$^+$ or *lexA*$^+$ *recA*1 genotypes. This indicates that cells possess a *lexA*$^+$-dependent error-free system for repairing alkylation damage. Unlike the *lex*$^+$-dependent error-prone repair pathway, this error-free system does not depend on an active *recA* gene product.

ACKNOWLEDGMENTS

Our work is supported by the Imperial Cancer Research Fund.

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THE UV INDUCIBILITY OF P32 SYNTHESIS

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ABSTRACT We have demonstrated that U.V. irradiation results in a markedly increased rate of synthesis of gene 32 protein. It is suggested that the production of gaps during repair of irradiated DNA is responsible for the stimulation of P32 synthesis. A model is proposed in which such regions of single stranded DNA compete for P32 by binding it non-specifically, thus reducing the amount of free P32. Because the expression of gene 32 is self-regulatory this would result in increased P32 synthesis. Additional observations suggest that gene 32 self-regulation operates at the level of translation and that P32 can inhibit the translation of g32 mRNA. Such a regulatory mechanism may be particularly well suited to the important role of P32 in repair of radiation damage.

INTRODUCTION

The product of bacteriophage T4D$^+$ gene 32 (P32) is essential for viral DNA replication, recombination, and repair. P32 binds tightly and cooperatively to single-stranded DNA and catalyzes DNA denaturation and renaturation under physiological conditions in vitro (Alberts and Frey, 1970). It has been demonstrated that when single-stranded DNA has bound P32 it becomes a much better template for DNA polymerase (Huberman et al., 1971) and is simultaneously rendered relatively insensitive to nucleolytic degradation (Curtis and Alberts, 1976). Alberts and Frey (1970) have suggested that the role of P32 in replication is that of stabilization of single-stranded regions which exist transiently near the growing point of a replicating DNA molecule.

In previous communications, we have presented evidence for an additional function of P32; that of regulation of its own synthesis. This evidence came from experiments in which

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cells were infected with phage mutant in gene 32 and the rate of synthesis of the defective P32 was measured. Using either missense or nonsense mutants in gene 32, we found that the expression of gene 32 was 5 to 10 times greater than in a wild-type infection. Similarly we found that infections in which a large amount of single-stranded DNA was generated also resulted in increased expression of gene 32. These observations formed the basis of a model for the regulation of expression of gene 32 in which single-stranded DNA and a regulatory site controlling P32 synthesis competed for available P32. The mutations in other genes which increased the expression of gene 32 were thought to act by causing the production of greater than normal amounts of single-stranded DNA. P32 was assumed to bind to this excess of single-stranded DNA, thus reducing the concentration of P32 available for repression of gene 32 expression.

In constructing this model for the control of gene 32 expression, we did not specify the level (i.e., transcription or translation) at which P32 acted to regulate its own synthesis. Two observations have been made, however, which seem more compatible with P32 self-regulation operating at the level of translation. High doses of uv result in a stimulation of P32 synthesis even though RNA synthesis is significantly inhibited. Moreover, the induction of new P32 synthesis is not affected by the inhibition of RNA synthesis with rifampicin.

MATERIALS AND METHODS

The procedures for labeling of infected cell proteins, for the preparation of lysates, and for determination of yields of specific proteins by gel electrophoresis have been described previously (Krisch and Van Houwe, 1976).

RESULTS

The Ultraviolet Light Stimulation of P32 Synthesis. Figure 1 shows the pattern of P32 synthesis in a lysis-defective infection (at comparable times the pattern of P32 synthesis in wild-type and tamA3 (lysis-defective) infections is identical). The synthesis of P32 commences early in infection and continues at a high rate until after the initiation of replication and of late gene expression, then begins to diminish. The u.v. irradiation of T4-infected E.coli at this time results in a rapid increase in the rate of P32 synthesis

(Fig. 1); no other T4-induced protein appears to have a similar response to irradiation. Even after a considerable delay, during which a nearly normal late protein synthesis pattern can re-establish itself, there is still a high rate of P32 synthesis.

A possible mechanism for the u.v. induction of P32 synthesis is suggested by our previous work (Krisch et al., 1974) on the control of P32 synthesis.

It is very likely that the immediate result of irradiation is an increase in gapped DNA generated by the repair mechanisms. Thus the "induction" of P32 synthesis may be explained as a result of the u.v. lesions generating binding sites for P32. Because P32 is self regulating, the intracellular concentration of free P32 could be reduced below the threshold level required to block further synthesis of this protein.

The effect of mutations on ultraviolet light inducibility of P32. With the above hypothesis in mind we have screened a number of mutants in genes thought to be involved in DNA replication, recombination and repair. (Fig. 1) Variation in the response might be expected if the mutation is in a gene which is involved in the generation or removal of P32 binding sites following irradiation. The results of these experiments indicate that no single mutation abolishes completely the stimulation of P32 synthesis following irradiation. Nevertheless, mutants in either gene 46 or 47 do markedly reduce it. These genes could code for the exonuclease which is involved in the exposure of single-stranded DNA in the region of the radiation lesion. The phenotype of a mutation in either of these genes is a lack of exonucleolytic activity, a reduction of recombination (Bernstein, 1968) and a greatly increased sensitivity to u.v. (Baldy, 1968).

The Induction of P32 Synthesis in the Absence of RNA Synthesis. Sauerbier et al. (1970) have observed that one of the effects of u.v. on T4-infected cells is to lower the rate of RNA transcription by causing the production of shorter RNA chains. Figure 2(a) demonstrates that such a dose of irradiation rapidly reduces incorporation of H^3-Uracil into RNA. Within several minutes incorporation is reduced to 25% of the level prior to irradiation. Addition of the RNA synthesis inhibitor Rimactan (100 µg rifampicin/ml) immediately after irradiation (within 10 s) reduced incorporation to less than 5% of the level in the untreated control infection (Fig. 2(a)).

In spite of this severe block of RNA synthesis, a high level of P32 synthesis is still induced in the irradiated Rimactan-treated culture (Fig. 2(b)). This result is compatible with a model of control of gene 32 expression which functions at the level of translation. Hence the P32 might bind to the gene 32 mRNA and hinder its translation. Additional evidence for the translational control of gene 32 expression is to be found in Russel et al., 1976 and Krisch et al., 1977.

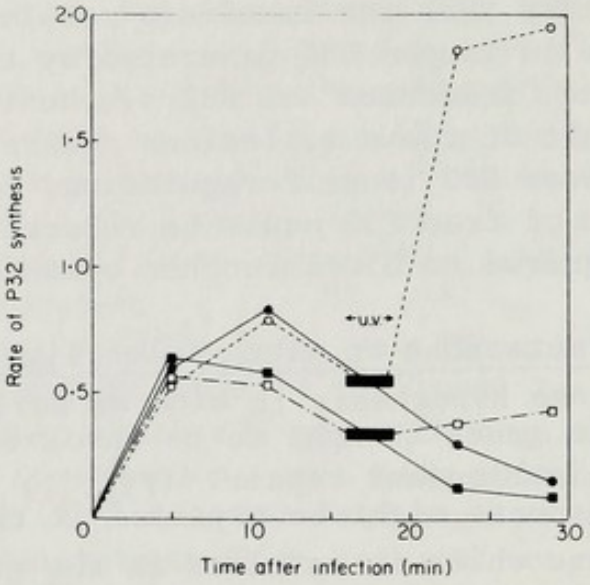

Fig. 1. Comparison of the rate of P32 synthesis in gene t-defective and gene 47-defective infection after u.v. irradiation.

-●-●-, tamA3-infected cells, no irradiation; -o-o-, tamA3-infected cells u.v. irradiated 16 to 19 min after infection (750 erg/mm^2); -■-■-, 47amA456-infected cells, no irradiation; -.-□-.-□-.-, 47 amA456-infected cells u.v. irradiated 16 to 19 min after infection (750 erg/mm^2).

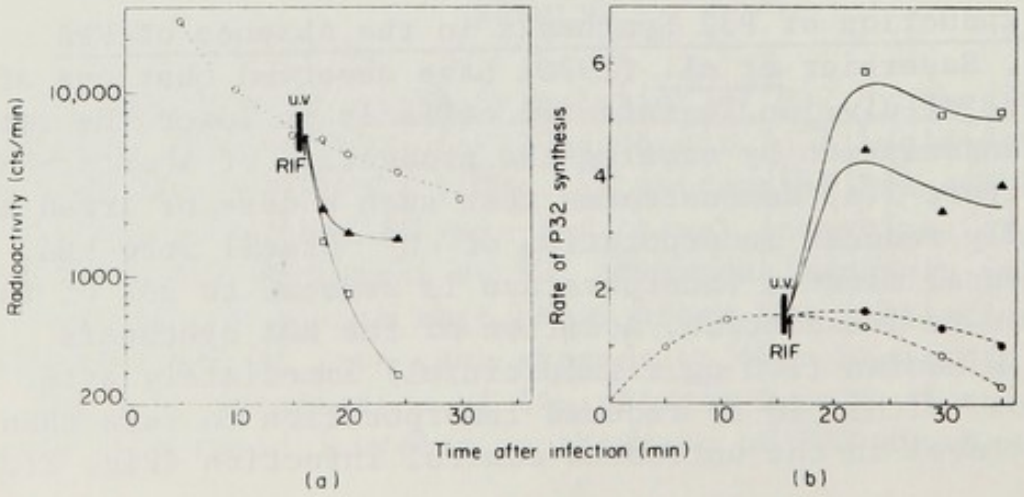

Fig. 2

Fig. 2. (a) RNA synthesis H^3-uracil incorporation by tamA3 infection after u.v. irradiation. At 16 min after infection the culture was split into 2 parts and 1 was irradiated. Immediately afterwards half of irradiated culture was treated with Rimactan (100 µg/ml)(RIF).

--o -- o--, tamA3-infected cells, no irradiation; -▲-▲-, tamA3-infected cells, u.v. irradiated 16.0 to 16.5 min after infection (600 erg/mm^2); -□-□-, tamA3-infected cells u.v. irradiated and then treated within 10 s with 100 µg Rimactan/ml.

(b) Comparison of the rate of P32 synthesis after u.v. irradiation and addition of Rimactan (RIF). E.coli AS19 at 2.0×10^8 cells/ml was infected at 30°C with tamA3 at a multiplicity of infection of 10.

--o--o--, tamA3-infected cells, no irradiation; --●--●--, tamA3-infected cells, no u.v., treated at 16.5 min after infection with Rimactan (100 µg/ml); —▲—▲—, tamA3-infected cells u.v. irradiated 16.0 to 16.5 min after infection (600 erg/mm2); —□—□—, tamA3-infected cells u.v. irradiated and then treated within 10 s with Rimactan (100 µg/ml).

ACKNOWLEDGEMENTS

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THE DEPENDENCE OF UV-MUTAGENESIS IN PHAGE T4 ON DNA LIGASE, POLYMERASE, AND NUCLEASE FUNCTIONS[1]

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ABSTRACT UV-induced reversion of two am mutations is undetectable if the phage have a temperature sensitive (ts) defect in their ligase (gene 30) function. If either a gene 43 DNA polymerase anti-mutator ts allele or a gene 47 ts allele defective in exonuclease function is present, UV-induced reversion of an am mutant is again undetectable. These results suggest that UV-induced lesions can be repaired by an error-prone pathway which employs a phage coded DNA ligase, polymerase, and exonuclease.

INTRODUCTION

UV has previously been proposed to induce mutations in bacteriophage T4 through the action of error-prone repair (1,2,3). Defects in genes x, y, and 1206 appear to lower this induced mutagenesis (2). Evidence is presented here that functional products of genes 30, 43, and 47 are also required for this process.

METHODS

Two amber alleles, amN52 and amS1, defective in late genes 37 and 52 respectively, were used to measure reversion frequencies. Double mutants were isolated from crosses of an am mutant to a ts mutant. The identities of all constructed strains were confirmed by complementation tests against the parental strains. Phage suspensions were placed beneath a General Electric germicidal lamp for various doses of UV light, diluted as necessary, and plated on E. coli (su 1^+) to

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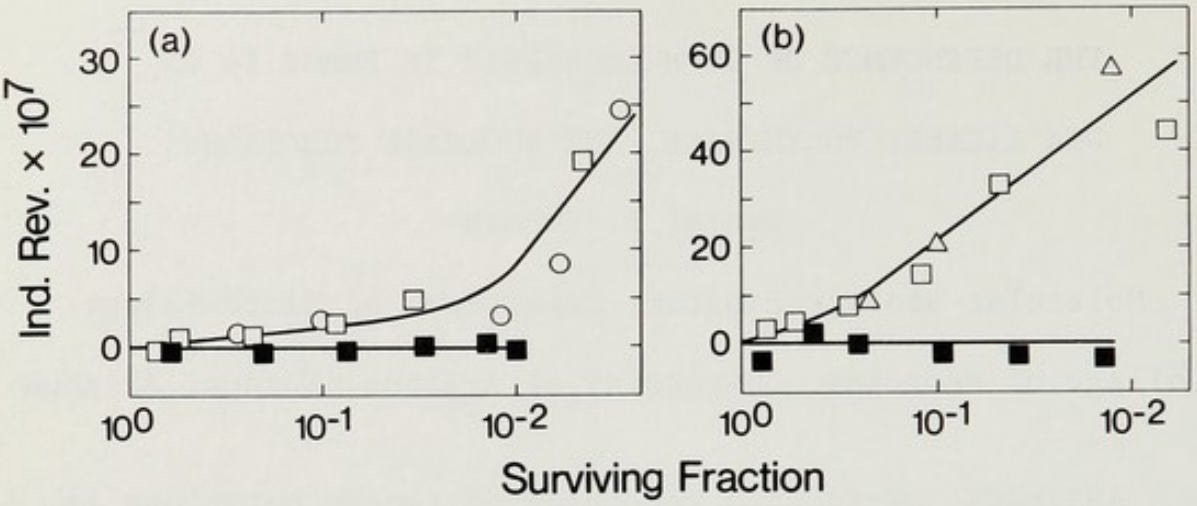

FIGURE 1. Frequency of newly induced revertants in am and am-tsB20 mutants as a function of surviving fraction after UV irradiation. The strain, the temperature of growth, the number of experiments averaged, the average spontaneous level of reversion x 10^{-7}, and the symbol are: (a) amN52, 31°, 2, 6.5, □ ; amN52tsB20, 31°, 2, 0.8, ■ ; amN52(segregated from tsB20), 31°, 2, 3.5, ○ . (b) amS1, 31°, 3, 15, □ ; amS1tsB20, 31°, 2, 9, ■ ; amS1tsB20, 25°, 2, 20, Δ .

determine survival of total phage and E. coli S/6/5 (su^-) to determine am^+ revertants. This direct plating procedure is described in more detail elsewhere (3). All experiments were repeated at least once and the data represent the average of multiple measurements.

RESULTS

The am and am-ts double mutants showed approximately wild-type UV sensitivity on E. coli CR63 at the temperatures used, except amN52tsL86, which showed increased UV sensitivity at 37°. The frequencies of induced revertants obtained at different surviving fractions are plotted for each strain.

The amN52 stocks used had an average spontaneous frequency of revertants of 6.5 x 10^{-7}. amN52 can be induced to revert above this level by UV, as shown in Figure 1(a) (open squares). In the presence of the temperature-sensitive gene 30 ligase allele tsB20, when growth after irradiation is at 31°, no UV-induced reversion is observed (filled squares). The amN52 allele, when segregated from the double mutant by crossing to wild type, again can be induced to revert by UV (open circles).

Figure 1(b) shows similar results for amS1. The single

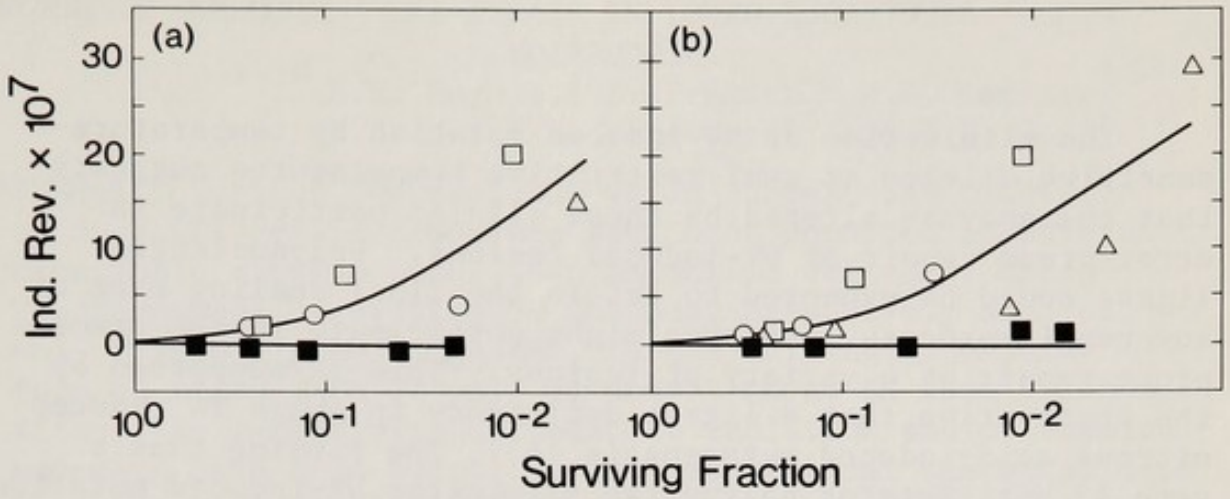

FIGURE 2. Frequency of newly induced revertants in amN52 and amN52-ts mutants as a function of surviving fraction after UV irradiation. The strain, temperature of growth, the number of experiments averaged, the average spontaneous level of reversion x 10^{-7}, and symbol are: (a) amN52, 37°, 4, 6.5, □ ; amN52tsCB120, 37°, 2, 1.6, ■ ; amN52tsCB120, 25°, 2, 1.4, Δ ; amN52(segregated from tsCB120), 37°, 2, 9.4, ○ . (b) amN52, 37°, 4, 6.5, □ ; amN52tsL86, 37°, 2, 1.6, ■ ; amN52-tsL86, 25°, 2, 2.5, Δ ; amN52(segregated from tsL86), 37°, 2, 6.7, ○ .

mutant amS1 shows an increasing frequency of induced revertants as the surviving fraction declines (open squares) when growth after irradiation is at 31°. The presence of the tsB20 allele eliminates UV-induced reversion (filled squares) at 31°. However, when growth is at 25°, amS1tsB20 shows inducible reversion (open triangles).

Figure 2(a) shows that amN52, when irradiated and grown at 37°, is induced to revert (open squares). In the presence of the temperature-sensitive anti-mutator allele tsCB120 no UV-induced reversion is observed at 37° (filled squares), even though a small increase could have been seen against a low spontaneous reversion frequency of 1.3 x 10^{-7}. At 25°, the double mutant does show UV-induced reversion (open triangle). The amN52 allele, segregated from the double mutant, also shows UV-induced reversion (open circles).

In Figure 2(b) the data indicating that amN52 is induced to revert by UV at 37° is again shown (open squares). In the presence of the gene 47 allele tsL86 at 37° no induced reversion in the double mutant is observed (filled squares), despite the low background reversion frequency of 1.6 x 10^{-7}. At 25°, however, the double mutant is induced to revert by UV (open triangles). The amN52 allele, segregated from tsL86, is also induced to revert by UV (open circles).

DISCUSSION

The elimination of UV-induced mutation by temperature-sensitive alleles at semi-restrictive temperatures suggests that the enzymes altered by these alleles participate in error-prone repair of UV-induced lesions. Polynucleotide ligase could be expected to act in the final sealing step of any repair process, and thus might participate in the error-prone repair of a variety of lesions. This is supported by the observation that a ligase deficiency in phage T4 reduced nitrous acid-induced mutagenesis (4). The finding that a gene 43 anti-mutator polymerase eliminates UV-induced mutation suggests that it is this replicative polymerase of the phage which is responsible for base mispairing in error-prone repair. This is similar to error-prone repair in E. coli, where the replicative DNA polymerase (pol III) was reported to be responsible for base mispairing in error-prone repair of UV-induced lesions (5). Gene 47 mutants are unable to convert nicks in duplex DNA into single-stranded gaps (6). The elimination of UV mutagenesis by a gene 47 allele suggests that if nicks in DNA are not expanded into gaps by the gene 47 exonuclease, error-prone repair is blocked.

Taken together, the data presented here suggest the outline of a pathway for error-prone repair of UV-induced lesions in phage T4. The UV lesions may stimulate the formation of nicks or short gaps in the phage DNA which are expanded by the action of the gene 47 exonuclease. The phage polymerase may then catalyze repair synthesis in a manner which results in a relatively high frequency of base mispairings. The polynucleotide ligase may then seal the final phosphodiester bond, resulting in duplex DNA which may contain a mutation.

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WORKSHOP SUMMARY: DNA REPAIR IN LOWER EUCARYOTES

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B.S. Cox,[4] E. Moustacchi[5] and J.B. Boyd[6]

SUMMARY: Various observations which suggest the existence of dark DNA repair processes have been reported in several eucaryotic species other than mammalian cells. Of these Saccharomyces cerevisiae is the most extensively studied, and Drosophila melanogaster, is the only multicellular form on which work is well advanced. About 55 loci which affect the sensitivity of yeast to radiation and/or chemical mutagens have been identified and statistical tests indicate that more are likely to exist. Some of the mutant genes show astonishing pleiotropism so that it is unlikely that all of these loci are involved primarily in repair. Studies with multiple mutants have revealed the existence of three epistatic groups associated with three categories of repair. One group contains no fewer than 9 loci which control pyrimidine dimer excision. The three groups may control the synthesis, assembly and coordination of three macromolecular complexes associated with repair, replication and recombination. Controlled excision of pyrimidine dimers from mitochondrial DNA does not occur in yeast; however dimers may be effectively bypassed by rapid and extensive recombinational events in mitochondrial DNA. In Drosophila over 20 genetic loci are known to affect sensitivity to radiations and chemical mutagens; 5 of these also influence female meiosis. Photoreactivation, pyrimidine dimer excision, repair replication and post-replication repair have been demonstrated in Drosophila, but all mutants so far examined exhibit a normal capacity to repair X-ray induced single strand breaks. The genetic versatility of organisms such as yeast and Drosophila, with their fast breeding cycles (involving meiosis as well as mitosis) and their system of cyptoplasmic as well as nuclear inheritance, makes them especially advantageous for enquiry into the general biological significance of the processes we identify with DNA repair.

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NARRATIVE DISCUSSION: The discovery of liquid-holding recovery in yeast after treatment with radiations and certain chemical mutagens (1,2) and of radiosensitive mutants in Ustilago (3), Aspergillus (4) and Schizosaccharomyces pombe (5) early indicated the existence in eucaryotes of processes analogous to DNA repair in bacteria (6). The first reports of radiosensitive mutants in S.cerevisiae came somewhat later (7,8), but soon it was found that a surprisingly large number of distinct genetic loci (~33) affect sensitivity to UV and X-rays (9-15) and also to methylmethanesulfonate (MMS) and nitrogen mustard (HN2) (16-18). More recently, in a screen for mutants sensitive to MMS, 17 further complementation groups have been found to confer sensitivity both to radiation and MMS and a further 5 to MMS only (19). In the work of both Cox and Parry (9) and of the Prakash's(19) statistical tests indicated that additional loci of each type exist.

Does this mean that as each new mutagen is used in such screens further loci will be found which confer sensitivity to radiation and chemicals? If this should be the case it could be argued that there exist, in addition to defective DNA repair many other independent, genetically controlled mechanisms through which cells can become sensitive to mutagens. Unfortunately we have no idea what these other routes to sensitivity might be, although permeability defects are an obvious possibility for chemicals.

Measurements of the sensitivity of various double and multiple RAD mutants have revealed the existence of three epistatic groups of loci which affect three categories of repair as well as other phenotypic characteristics (18, 20-25). These three groups are named, as a matter of convenience, by one of the prominent loci in each: the RAD3 group controls dimer excision (26, 27); the highly pleiotropic RAD6 group controls UV-induced mutagenesis and some of the loci in this group are known to be required for the successful completion of meiosis and for resistance to the anti-folate drug trimethoprim (28, 29); and the RAD52 gene of the third group controls the repair of X-ray induced double strand breaks (30,31). In these proceedings Louise Prakash reports finding three more loci (RAD7, RAD14 and MMS19), in addition to the six previously known (RAD1, 2, 3, 4, 10 and 16), which are defective in their ability to remove endonuclease sensitive sites or dimers, from their nuclear DNA. Even more perplexing is Prakash's finding that the cell division cycle mutant cdc8, which is slightly UV sensitive may belong simultaneously to both the RAD3 and RAD6 groups. (In unpublished studies

F. Eckardt has found that the mildly UV-sensitive mutant r_1^s, which as yet has no RAD designation, is epistatic both to RAD2 and RAD51, and so it appears to belong to two different epistatic groups.) Despite the increasing number of loci which appear to be involved in repair,it is unlikely that more than three epistatic groups of such loci will be found. This surmise is based on the observation that in haploid triple mutants containing one allele from each group, the UV dose for one lethal hit (D_{37}) produces only 1 or 2 dimers; thus it seems unlikely that any additional processes for the repair of UV damage exist in haploid strains (22, 32).

We are left with a picture of a large number of loci which fall into three, possibly overlapping, epistatic groups which can be associated with three modes of repair. Some of these loci, especially those in the RAD6 and RAD52 group are surprisingly pleiotropic and affect such diverse functions as spontaneous (33-35) and induced mutagenesis (24,28,36,37,) recombination (38-42), meiosis and spore viability (43, 44), and sensitivity to the anti-folate drug trimethoprim, which, among other things, blocks de novo synthesis of certain DNA precursors (45). All of this suggests that we should re-examine, and possibly enlarge, our view of the molecular significance of these epistatic groups. It is customary to think of them as corresponding to distinct 'biochemical pathways' for repair, and certainly structural genes for the enzymes involved in sequential steps of repair would be expected to belong to the same epistatic group. However, the fact that the group for dimer excision is known to contain at least nine loci suggests that a substantial number of gene products are required for the control and coordination of this process within the cell. Thus it is possible that our three epistatic groups correspond not merely to three enzymic reaction sequences, but more broadly, to three complex, and functionally coordinated macromolecular structures, which are required to effect and properly control in time and space the three major manifestations of DNA metabolism, viz., excision repair, replication (possibly including some steps of precursor synthesis) and recombination. If such 'metabolic complexes' exist then it is not surprising that there should be a substantial number of complementation groups epistatic to one another; and if these complexes normally must interact for proper function during the mitotic and meiotic division cycles, then it is also not surprising that there should exist some loci which only weakly affect radiation sensitivity but which belong to two or more of these groups.

Not only has dimer excision been demonstrated biochemically (26, 27, 46), but in addition, the difference between the shouldered UV survival curve of an excision proficient strain and the corresponding exponential curve of an excision deficient mutant has been accounted for quantitatively on the basis of the decline in dimer excision efficiency with increasing dose (32, 47).

Recently at York University, Orna Goldberg and J.G. Little have succeeded in demonstrating repair replication in a Pettijohn-Hanawalt assay after UV irradiation of a ρ_o haploid strain auxotrophic for dTMP (such strains can be density labeled with dBUMP (48)).These initial findings suggest that the genetic control of repair replication can be studied readily in tmp auxotrophs; in addition these strains, some of which are stable grandes, afford the opportunity for investigating DNA strand exchanges in recombinational episodes at both the nuclear and mitochondrial levels (49). The discovery and characterization of two classes of tmp auxotrophs, the first presumptively defective in thymidylate synthetase, the second in folate metabolism, provides the best solution to what has been a major technical barrier in the study of DNA metabolism in yeast, namely, the inability to label specifically yeast DNA with exogenous radioactive precursors (49-51). In addition, such auxotrophs can be used for the study of 'thymineless' death and mutagenesis (52, 53).

One of the many advantages of the yeast system is that one can study repair processes that are dependent on the presence either of sister chromatids or homologous chromosomes. In these proceedings, Michael Resnick shows that X-ray induced double strand breaks can be repaired provided the RAD52 gene product and either haploid G-2 sister, or diploid homolog, DNA molecules are present. It had previously been shown that unrepaired double strand breaks are lethal (30,31) and can be expressed dominantly (54). It is possible to explain the X-ray resistant 'tail' caused by budding cells in haploid cultures (55) as well as the variations in X-ray sensitivity during the cell cycle of synchronized haploid cultures on the basis of double strand break repair. Unfortunately, the relation between repair and recombination is far from clear, for as Brian Cox points out in these proceedings UV induced recombination does not contribute to survival in yeast, whereas it does in Ustilago; however, as is apparent from Resnick's contribution X-ray induced recombination does contribute to survival.

In yeast error-prone repair (EPR) is controlled by

loci of the RAD6 epistatic group. The low dose linearity of UV mutagenesis indicates that there must exist a constitutive component of EPR; at higher doses reversion induction rises as the square or sometimes higher powers of dose. This dose behaviour could arise from δ-effects (56), from inhibition of error-free repair or from an induced component of EPR. Calculations indicate that in RAD wild-type strains the latter explanation is the more likely (57). The phenomenon of radiation induced UV-resistance also might be caused by induced components of repair (58); and Fabre and Roman recently demonstrated the existence of radiation induced transmissable factors capable of enhancing recombination in diploids (59). In these proceedings Eckardt, Moustacchi and Haynes report preliminary experiments which indicate the existence of a cyclohexamide sensitive component of EPR which again would be consistent with the presence in yeast of inducible error-prone-repair.

The mitochondrial genome of yeast provides an additional system of great evolutionary and metabolic significance for the study of repair, replication and recombination as well as nucleocytoplasmic relations. In these proceedings Ethel Moustacchi presents evidence indicating that pyrimidine dimers in mDNA may be bypassed effectively by extensive recombinational turnover of the mitochondrial genome (60,61). Dimers in mDNA are photoreactivable but they are not excised in a controlled fashion (46, 62); rather, partial degradation of mDNA occurs which is the greatest during liquid-holding of UV-irradiated stationary phase cells. However, during liquid-holding of irradiated exponential phase cells there is a partial recovery of ρ^+ genotypes and marker rescue in the remaining induced ρ^- clones. In addition, nuclear or mitochondrial mutants UV sensitive to ρ^- induction exist (63). Thus, despite the lack of normal dimer excision, the possibility of an accurate repair mechanism for mDNA in growing cells cannot be ruled out.

Studies on repair in Drosophila are beginning to reveal interesting similarities with those in yeast,in particular, the existence of a large number of loci which affect mutagen sensitivity together with meiosis, chromosome breakage and recombination. In these proceedings James Boyd summarizes this work and points out that of the 20 loci which affect mutagen sensitivity, 5 also affect female meiosis; this latter class of mutants provides a good opportunity to study meiotic recombination functions in somatic cells.

In his verbal summary of the workshop, R.H. Haynes confessed a rather visceral worry over the anthropocentric overtones that may associate with the use of the word 'repair'

in molecular biology: we say the cells are repairing DNA after irradiation; they may be supporting some larger, unitary process of which dark repair is as much an artefact as a functional homeostatic response. Replication, transcription, recombination and repair might not appear so complicated if we could envision the machinery that effects them.

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PATHWAYS OF DNA REPAIR IN YEAST[1]

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ABSTRACT The *RAD1* excision-repair pathway of yeast consists of three additional genes, *RAD7*, *RAD14* and *MMS19*, all of which are shown to be defective in excision of pyrimidine dimers. In addition, two genes, *MMS3* and *CDC8* are discussed in terms of their effect on UV-induced mutations.

INTRODUCTION

A large number of radiation-sensitive (*rad*) mutants has been isolated in the eucaryotic microorganism, the yeast *Saccharomyces cerevisiae* during the past ten years[1-4]. At least 32 genetic loci have already been identified among these numerous *rad* mutants. Comparisons of sensitivities to killing of various double mutant *rad* combinations indicate that three pathways exist for repairing UV-induced damage in DNA. One group of mutants consists of 6 genes, *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD16*, involved in excision of UV-induced pyrimidine dimers [5-11]. Mutants at any of these 6 loci show enhanced UV-induced mutations compared to the excision-proficient parent strains[12]. Similar observations have been made in the excision-defective mutants of *Escherichia coli*[13]. The second epistatic group in yeast consists of 7 loci involved in error-prone repair of UV-induced damage: *RAD6*, *RAD8*, *RAD9*, *RAD18*, *REV1*, *REV2* and *REV3*. All of these mutants show reduced UV mutability for at least some, if not all, loci tested[12,14]. In addition, some of them have pleiotropic effects on spontaneous mitotic gene conversion and spontaneous mutation.

In *E. coli*, the sensitivity of a double mutant consisting of *uvrA* and *recA* is such that one dimer is sufficient to kill the cell, indicating that no other pathways play a major role in repair of UV damage[15]. In yeast, such comparable sensitiv-

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ity is achieved only in a triple mutant[16] consisting of one member of each of the two epistatic groups mentioned above, as well as a member of the third group, mutants of the *RAD50* to *RAD57* loci. These mutants were selected for sensitivity to X-rays and do not confer marked sensitivity to UV, unless in combination with a *rad3* mutant, defective in excision repair[4,16]. Thus, in yeast, the triple mutant in which all three pathways are blocked is as sensitive as the double mutant *uvrA recA* in *E. coli*.

The presence of a third repair system in yeast as opposed to two in *E. coli* increases the complexity of repair processes. In addition, the genetic control of repair in yeast involves a large number of loci. In this study, we present evidence for an even greater complexity. We report on 3 additional loci, 2 *RAD* loci and 1 *MMS* locus (mutants selected for sensitivity to methyl methanesulfonate, MMS[17]) involved in excision repair, and 2 more loci, *MMS3* and *CDC8*, involved in error-prone repair of UV damage.

MATERIALS AND METHODS

Experimental methods used for measurements of excision ability and mutation induction were as previously described[6,18].

RESULTS AND DISCUSSION

Cells of the *rad7* mutant irradiated with an incident fluence of 100 J/m^2 (7.3% survival) were either lysed immediately after irradiation or incubated in the dark in growth medium for 4 hours. Nuclear DNA was purified by CsCl centrifugation and sedimented in alkaline sucrose gradients either with or without prior treatment with a UV-specific endonuclease activity obtained from partially purified extracts of *Micrococcus luteus*. DNA from unirradiated cells does not contain any endonuclease sensitive sites. Treatment of DNA from both irradiated cells and irradiated cells given a dark repair period shows that endonuclease sensitive sites are still present. The DNA from irradiated cells given no repair period has a molecular weight of about 6.3 X 10^5 while that from irradiated cells given a 4 hour dark repair period has increased to about 1.5 X 10^6, suggesting that most of the endonuclease-sensitive sites, or dimers, are still present, since DNA from unirradiated cells has a molecular weight of about 1.1 X 10^7. No further decrease in endonuclease sensitive sites is observed in cells irradiated at a fluence of 50 J/m^2 (41.5% survival) and given a 4 hour dark repair period. A similar experiment was carried out with a *rad14* mutant irradiated at 100 J/m^2 (0.27% survival). Under these conditions, all endonuclease sensitive

sites were retained in the DNA even after a 4 hour dark repair period. On the other hand, all detectable sites were removed from the nuclear DNA of a *rad6* mutant irradiated at a fluence of 100 J/m^2, resulting in less than 0.01% survival[7]. The third mutant analyzed in this way which showed a defect in dimer excision was the *mms19* mutant, originally isolated as a strain sensitive to MMS. The *mms19* mutant irradiated at a fluence of 100 J/m^2 (11.5% survival) retained endonuclease-sensitive sites in nuclear DNA following a 4 hour dark repair period sufficient to result in a loss of such sites from either a *RAD+* (wild-type) or *rad6* mutant. Thus, all three mutants, *rad7*, *rad14* and *mms19*, are defective in their ability to remove endonuclease sensitive sites, or dimers, from nuclear DNA. All three mutants show enhanced UV-induced mutation[12,19], a phenotype characteristic of both the other yeast mutants which are defective in excision of dimers as well as the *E. coli* mutants with this defect. Double mutant analysis of *rad7* and *rad1*, and *rad14* and *rad1* strains indicates that the *RAD7* and *RAD14* genes fall in the *RAD1* pathway. In addition, preliminary results indicate that the *MMS19* gene is also in the *RAD1* pathway. Thus, in yeast there are now nine distinct genetic loci, all involved in one pathway of excision of UV-induced pyrimidine dimers. Perhaps only one or two loci are the structural genes for the yeast UV-endonuclease(s) while the others may be more indirectly involved in dimer excision.

We have uncovered two additional mutant loci which seem to belong to the error-prone repair pathway of yeast. One of these, *MMS3*, is being studied by P. Martin in our laboratory. The *mms3-1* mutant is sensitive to MMS and UV in haploids. The *mms3-1* haploids are no more sensitive to X-rays than the wild type haploids. Homozygous *mms3-1/mms3-1* diploids, however, are much more sensitive to MMS, UV and X-rays than the heterozygous *mms3-1/+* or homozygous *+/+* diploids. Preliminary experiments on induced mutation indicate that UV-induced reversion of several markers is lowered in homozygous *mms3-1/mms3-1* diploids but not in haploids. EMS-induced reversion, on the other hand, is reduced in both *mms3-1* haploids and *mms3-1/mms3-1* diploids. UV-induced mitotic prototrophy has been examined in *his1-1/his1-315 mms3-1/mms3-1* diploids and a marked reduction is observed. The molecular basis for the defect in this mutant is under current investigation.

The cell division cycle mutant *cdc8*, a temperature-sensitive mutant defective in DNA chain elongation at the restrictive temperature (36 C)[20], was found to diminish UV-induced reversion of *lys2-1*. Although *cdc8* strains are somewhat UV-sensitive, reduced reversion is observed even at fluences yielding 20-30% survival in *cdc8* haploids. In all the reversion studies, at least 4 strains of each genotype were exam-

ined. At a UV fluence of 50 J/m^2, there is about a 10-fold reduction in *lys2* revertants compared to *CDC+* strains. All three alleles tested, *cdc8-1*, *cdc8-2* and *cdc8-3*, show this effect of decreased reversion. Three other markers tested, *arg4-17*, *tyr1* and *ura1* also exhibited greatly reduced UV revertibility in *cdc8* haploids. In order to determine whether the *cdc8* mutant belongs to the error-prone repair pathway, the sensitivity to killing by UV was examined in *cdc8 RAD+*, *CDC+ rad6*, *CDC+ RAD+* and *cdc8 rad6* haploids. Results indicate that *cdc8* and *rad6* belong to the same epistatic group. However, double mutant analysis with *rad1* strains reveal that *cdc8* belongs to the *RAD1* pathway as well. Studies are under way to determine if the effect of *cdc8* on induced mutation is direct, that is, it functions in the error-prone pathway, or whether its effect is indirect.

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THE IMPORTANCE OF DNA DOUBLE-STRAND BREAK REPAIR IN YEAST

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ABSTRACT Recent work of several investigators on the repair of ionizing radiation damage are summarized. Double-strand breaks, the major lethal lesions, are repaired in all yeast cells except in haploids prior to DNA synthesis (G-1). Complete repair requires duplicated chromosomes and the RAD 52 pathway and may be similar to that in higher eukaryotes. The RAD 52 pathway is also required in radiation-induced recombination and during meiosis.

The yeast Saccharomyces cerevisiae is an ideal eukaryote for studying the effects of DNA damaging agents and repair since there is the potential for relating biochemical changes to induced genetic events such as recombination, and mutation as well as survival. In addition to being able to isolate sensitive mutants readily, recent advances have enabled the sorts of biochemical analysis obtained in other systems including the detection of repair synthesis (1), DNA degradation (2), single- and double-strand breaks (3,4,5,) and pyrimidine dimers (6,7,8,9). Mechanisms involved in repair can be examined for their role in normal cell growth and meiosis. It is also possible to measure repair during the cell cycle using various synchronization techniques (10) or cell cycle mutants (11).

The response of yeast to ionizing radiation has appeared to be unique among all prokaryotes and eukaryotes examined. There are two populations--sensitive and very resistant--in a culture of haploid yeast, whereas diploid cells are generally resistant to X-rays (12). The resistant population correlates with the presence of budding cells (12) and more specifically those cells which have undergone DNA synthesis (13). Based on genetic and biochemical investigations discussed below these observations are due to a mechanism of DNA double-strand break (DSB) repair (which does not exist in E. coli) that can act in all but G-1 haploids.

The neutral sucrose gradient techniques of Petes and Fangman (14) have been adapted by Ho (4) and Resnick and

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Martin (5) to monitor the induction and repair of DSB in growing yeast cells. Based on their results the induction of DSB in yeast is a) linear, b) independent of ploidy and c) similar to that in other organisms including human cells (15). Repair is rapid and at low doses is complete within 3 to 6 hours. To some extent protein synthesis is required; however, small doses do not appear to induce the repair process (Resnick, unpub.). At higher doses (>50 krad) the repair appears to saturate (4), possibly because of an accumulation of DSB which might be unrepairable or a limited capacity for repair. Similar observations have been made with CHO cells at comparable doses (16).

It is clear from experiments with rad 52 mutants that unrepaired DSB can be lethal. Mutants in the RAD 52 pathway (17) are a) extremely sensitive to ionizing radiation (comparable to G-1 haploids), b) lack DSB repair (4,5) and c) there is a correlation of 1 to 2 DSB per lethal event (5). [Single-strand breaks are repaired in rad 52 mutants (Resnick, unpub., and Cox pers. com.).] These lesions are expressed dominantly when unrepaired (18). If irradiated rad 52 cells are mated with unirradiated rad 52 cells, the resulting zygotes die, while if crossed to unirradiated wild type cells the zygotes will most likely survive. Although the sensitivity of diploid (5) and higher ploidy rad 52 strains (18, 19) can thus be attributed to the dominant effect of unrepaired breaks, the mode of lethal expression remains unanswered. Chromosome, or chromatid bridges, and DNA degradation have been suggested (4,5,18). The latter possibility is unresolved since degradation has not always been observed when DSB remain unrepaired (see below).

Prompted by the above studies, DSB repair and survival have been examined during the cell cycle of haploid and diploid cells. Luchnik, et. al., (20) have determined the liquid holding recovery of survival and the corresponding DSB repair in stationary (G-1) diploids and haploids. Survival after 100 krad of the former increases when cells are held in buffer while the latter are unaffected (20,21,22). Correlated with this is DSB repair in diploids but not haploids after 48 hours in non nutrient medium. Therefore, the G-1 sensitivity of haploids can be attributed to a lack of DSB repair.

Brunborg and Williamson (23) have fully described the sensitivity during the cell cycle using a strain which exhibits exceptional synchrony (24). Resistance is acquired during DNA synthesis and is maintained at a high level throughout G-2. During mitosis the resistance drops rapidly and remains low in the subsequent G-1 stage. The high resistance in G-2 cells corresponds to efficient DSB repair (25).

In the subsequent G-1 period DSB repair is absent and there is DNA degradation (similar to the results of Ho (4) with a rad 52 mutant but unlike those of Luchnik, et. al. (20), with G-1 haploids or Resnick and Martin (5) using a rad 52 diploid). From these results it appears that DSB repair and resistance to ionizing radiation requires preexisting duplicated chromosomes, (sister chromatids or homologous chromosomes) and the RAD 52 pathway gene products. Based on the results with G-2 cells semiconservative replication is not required for repair.

During the latter experiments, the DNA from sucrose gradients was examined for recombinant structures (26) with the electron microscope. Less than 1% of the molecules recovered from control and irradiated only G-2 and G-1 cells were forked (—<) or branched (><). Postirradiation incubation did not increase the frequency in G-2 cells; however, 9% of the molecules recovered from incubated G-1 cells had forked structures (—<). Since these could be detected in the absence of DNA synthesis, they could have resulted from an interaction between DNA molecules in the cell (abortive recombination?).

The role of DSB repair or at least the RAD 52 pathway (17) has also been examined with regard to mutation and meiotic events. Magni, Panzeri and Sora (27) have demonstrated that X-ray induced mutation is the same in G-1 and G-2 haploids indicating that DSB repair is not required. However, the RAD 52 gene product appears to be involved in other types of mutations since both spontaneous (28) and chemically induced (29) mutagenesis are alterred in rad 52 mutants.

The RAD 52 gene product is also essential during meiosis. Mutants in the RAD 52 pathway can undergo several steps in meiosis and yield the expected four spore products; however, all the spores are inviable (30). The events associated with cell death occur after the premeiotic round of DNA synthesis. Since meiotic recombination [detectable without the completion of meiosis (31)] and radiation induced mitotic recombination (32) are not observed, the RAD 52 pathway is probably involved in or at least monitors recombinational events. Possibly the two observations are related to the repair of DSB although there is no evidence for DSB's during meiosis in yeast (33).

As mentioned above, the survival of haploid yeast appears anomalous when compared to other eukaryotes. Recently it was shown that Schizosaccharomyces pombe (34) also has a sensitive G-1 and a resistant G-2 stage. Unfortunately, it has not been possible to establish whether the G-1 phase of haploid eukaryotes are generally sensitive although all fungi are resistant in the G-2 phase. Surprisingly, when the data

from lower eukaryotes is compared (35) to several vertabrate cell lines in terms of lesions/cell at 10% survival, the sensitivities are similar (within a factor of 10 while DNA contents differ by as much as 1000 times). Since DSB repair is generally observed in diploid mammalian cells (see 15) and also during the G-1 and G-2 phases of the cell cycle (in CHO cells (36)) the effects and the repair of DSB may be similar in this wide variety of organisms.

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ON THE INDUCIBILITY OF ERROR-PRONE REPAIR IN YEAST

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ABSTRACT: Preliminary results from two differently designed experiments are consistent with the existence in yeast of inducible, as well as constitutive components of error-prone repair. A split-dose experiment shows that both UV mutability and resistance to killing are enhanced in cells liquid-held for 24 hours after an initial 'conditioning' dose of UV; these effects are blocked by the presence of cycloheximide during the liquid-holding period. An acute dose experiment shows that if irradiated cells are liquid-held for 3 days in the presence of cycloheximide, then mutation frequencies at higher doses are reduced such that the normal quadratic component of the reversion induction curve is virtually eliminated and the observed frequency increases linearly at all doses.

If *RAD* wild-type strains of *Saccharomyces cerevisiae* are irradiated with UV, and then held in an appropriate non-nutritive medium for prolonged periods before plating, substantial non-inheritable increases in both survival and UV resistance can be observed (1). Limited protein synthesis occurs during post-irradiation liquid-holding even in auxotrophic cells; this synthesis can be blocked by cycloheximide (CYH) (2,3), a specific inhibitor of cytoplasmic protein synthesis (4). In a diploid strain recovery after UV is unaffected by cycloheximide for holding times less than about 12 hours; for longer times recovery is inhibited and ultimately viability is lost even though CYH is non-toxic to unirradiated cells held under the same conditions(5).

In experiments at Orsay a split-dose protocol was used to monitor *HIS1* revertants as well as survival both in the presence(5 µg/ml) and absence of CYH. Log phase cells of the haploid *RAD* wild-type strain MH32-6d (α *ade2 his1*) were harvested after 12 hours growth in supplemented minimal medium and resuspended for irradiation and liquid-holding (28°C with constant aeration) at a titer of 6×10^7 cells/ml in triple distilled water; the cultures contained about 25% budded cells. As shown in the table this strain shows liquid-holding recovery (cf. columns 2 and 3) and enhanced UV resistance after an inital UV dose of $50 J/m^2$ and 24 hours liquid-holding (cf. columns 2 and 5); in the presence of CYH both liquid-holding recovery and the enhancement of UV re-

sistance after holding are abolished (cf. columns 2,4 and 6) Reversion frequency is reduced in this strain by liquid-holding (cf. columns 7 and 8), but there is an increase in mutations induced by subsequent UV doses (cf. columns 7 and 9) and this increase is abolished if CYH is present in the holding medium (cf. columns 9 and 10).

Dose	SURVIVAL					MUTANTS/10^5SURVIVORS			
J/m^2	2	3	4	5	6	7	8	9	10
25	80	98	72	-	-	16	5.5	-	-
50	70	90	59	-	-	20	9.5	-	-
75	-	-	-	80	27	30	11	46	13
100	17	21	7.9	40	6.5	46	12	60	16
150	2.3	5.0	.98	10	1.5	60	16	98	20
200	.35	1.6	.19	3.2	.35	58	20	84	10

Columns 2, 7 = immediate plating; 3, 8 = 24 hrs LH;
4 = 24 hrs LH+CYH; 5, 9 = 50J/m^2 + 24 hrs LH;
6, 10 = 50J/m^2 hrs LH+CYH.

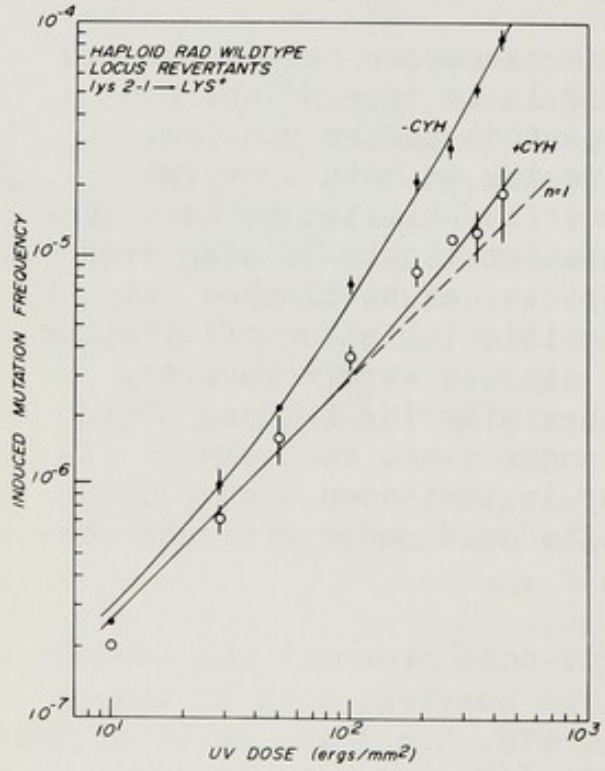

Figure 1

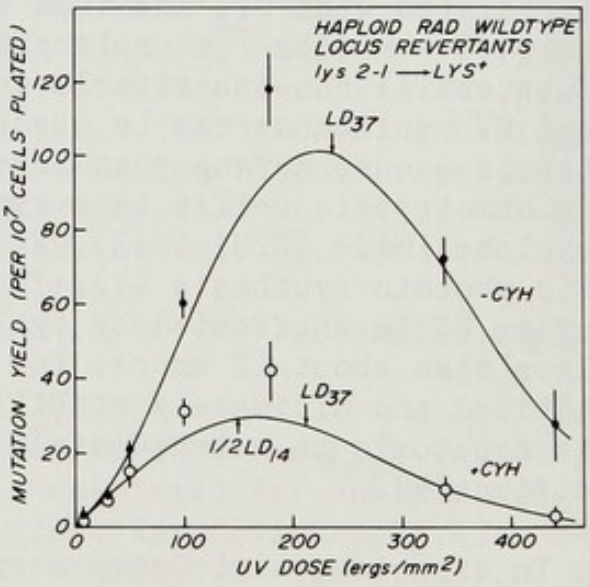

Figure 2

In experiments at Toronto *LYS2* revertants were measured in the haploid *RAD* wild-type strain HT4-21A (α *adel lys2 his3*) in 4 day stationary phase cells (no budding cells discernable). After irradiation the cells were held at 30°C under non-growth conditions in 0.67% yeast nitrogen base without amino acids at a titer of 10^8/ml both in the absence and presence of CYH (20 µg/ml) for 3 days prior to plating. Under these conditions both survival and mutation induction are the

same as for immediate plating. The cells held in CYH are sensitized slightly by this treatment. The induction kinetics in the absence of CYH are biphasic linear-quadratic (Fig.1); as in bacteria, the $(dose)^2$ part could arise from a UV-inducible mode of error-prone repair (6). In the presence of CYH the quadratic component is largely abolished. Mathematical analysis of the induction kinetics suggests that the two-hit component arises from dose dependent induction (or activation) of error-prone repair rather than the inactivation of error-free repair or the preferential survival of prototrophs as compared with auxotrophs.

Figure 2 shows mutational yield data (corrected for spontaneous background) both in the presence and absence of CYH. The curves were calculated theoretically using parameters obtained from best fits of the survival and mutation frequency data. Calculations show that in the absence of CYH the maximum yield is almost four times too large to be explained by preferential survival of the mutants (δ- effects, ref.7), but it is consistent with the presence of a two-hit component arising from some UV induced mutagenic mechanism. The yield curve in the presence of CYH is essentially as expected for linear mutation induction and a shouldered survival curve (maximum yield should be at one half the LD_{14} dose for such kinetics).

Despite differences in experimental design and the kinetic behaviour of the strains used, these two experiments, together with our mathematical analysis, make it reasonable to embark on a direct biochemical search for inducible components of error-prone repair in yeast. Radiation induced transmissable factors which enhance recombination already have been demonstrated (8).

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THE FATE OF MITOCHONDRIAL DNA AND OF MITOCHONDRIAL GENETIC MARKERS AFTER UV IRRADIATION OF *Saccharomyces cerevisiae*

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ABSTRACT UV-irradiation of yeast cells at all growth stages leads to a degradation of the mitochondrial DNA. This effect is predominant for dark held stationary phase cells and is accompanied by an increase in ρ^- mutants with a loss of mitochondrial markers in the ρ^- population. In exponential phase cells the degradation is biphasic with a slow rate component for high doses and long periods of dark holding. Repair processes of the recombinational type are likely to take place in this case since a partial recovery of the ρ^+ genotype and a rescue of markers in the remaining ρ^- clones are observed.

INTRODUCTION

A controlled excision of UV-induced pyrimidine dimers does not operate on mitochondrial DNA (mDNA) in yeast cells (1, 2). However, the fate of the respiratory genetic determinant (ρ) is not irrevocably determined after a UV treatment since a) nuclear or mitochondrial mutants UV sensitive to ρ^- induction have been identified (for review, see 3) dark liquid holding (LH) of UV treated exponential phase cells is followed by a reduction in the frequency of ρ^- mutants (4) c) inhibitors of protein synthesis (5,6) interfere with the LH response of the ρ^- induction. The possibility therefore of an accurate repair mechanism of UV damaged mDNA, at least for growing cells, is still open.

MATERIAL AND METHODS

For media, definition of growth stages, UV irradiation, LH in non growth medium and detection of ρ^- mutants see ref. 4.

For radioactive labelling, DNA preparation, equilibrium and velocity gradients, see ref. 7.

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The genetic analysis of mitochondrial markers in ρ^- clones was performed according to Deutsch *et al.* (8) for antibiotic resistance and according to Slonimski and Tzalgaloff (9) for the mit^- (deficiencies in mitochondrial proteins).

RESULTS AND DISCUSSION

The Fate of mDNA After UV Irradiation. LH of UV-treated stationary phase cells leads to an extensive loss of mDNA (1, 2) and to an increase in ρ^- mutants relative to an immediate plating (4). However for exponential phase cells LH leads to a degradation of mDNA only during the first 24 hrs. followed by a stabilization maintained until 40 hrs (Table I). This is accompanied throughout by a decrease in the ρ^- population (4 and Table III). The dose response for both effects is biphasic with a sharp rise at low doses and a slow increase at dose above 100 J/m^2 (Table II).

TABLE I

THE FATE OF mDNA AS A FUNCTION OF TIME AFTER A DOSE OF 100 J/m^2.

Time (in Hrs)	0	2	5	17	20	24	40
% Degradation	0	15	39	48	48	50	50

TABLE II

DEGRADATION OF mDNA AS A FUNCTION OF UV DOSE AND AFTER 40 HRS LH.

Dose in J/m^2	0	25	50	100	200
% Degradation	0	20	40	52	62

The Fate of mitochondrial markers in the ρ^- population. When UV treated stationary phase cells are plated immediately (table III) a biphasic response is seen for both the induction of ρ^- and for loss of markers in the ρ^- population. In the low dose range the induction of ρ^- is associated to a higher retention of markers. For higher doses the loss of markers increases linearly. After LH this biphasic pattern is retained; for high doses both the frequencies of ρ^- and of markers loss increase. The extensive degradation of mDNA (1) is consequently associated

TABLE III

UV INDUCTION OF ρ^- MUTANTS AND % LOSS OF MARKERS ON IMMEDIATE (IMM) PLATING AND AFTER LH (40 HRS.)

ose /m²	STATIONARY PHASE												EXPONENTIAL PHASE											
	% ρ^-		170°		C°		E°		990°		O°_{II}		140°		% ρ^-		170°		C°		E°		990°		O°_{II}		140°	
	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH	IMM	LH
	4 (2.3)	4 (2)	38	41	42	46	42	41	39	34	48	49	61	52	7 (4.7)	8 (1)	54	41	57	45	53	45	47	45	67	61	67	59
5	17 (7)	26 (6)	21	41	33	41	31	42	24	39	49	35	56	49	18 (5.7)	13 (3)	24	24	34	33	33	24	8	19	44	28	70	23
50	28 (7.5)	39 (29)	27	32	35	35	33	32	26	32	29	50	45	71	35 (7.3)	20 (7.3)	9	18	17	30	13	22	42	31	50	40	50	46
75	44 (24)	68 (68)	29	48	39	55	38	53	40	46	57	66	69	85	45 (12)	28 (15)	22	21	33	27	32	24	41	29	52	50	83	74
100	60 (45)	88 (86)	41	60	48	64	47	63	47	61	64	76	79	89	67 (28)	48 (30)	51	35	50	39	41	30	48	39	68	59	91	67
150	72 (71)	93 (94)	47	72	59	80	56	78	55	81	73	83	84	89	75 (68)	65 (57)	52	50	57	57	51	50	61	46	71	66	99	79
175	78 (77)	97 (98)	56	82	62	91	60	87	63	87	77	85	90	97	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The treated haploid strain MH 32-6d (α ade_2 his_7) carries three drug resistance marker C^R, E^R, O^R_{II} (mitochondrial mutations conferring resistance to chloramphenicol, erythromycin and oligomycine).[7] For the detection of drug resistance in ρ^- a drug sensitive ρ^+, 1073 ($aleu_3$) was used. The loss of the mit^+ functions was detected by crosses of the ρ^- mutants derived from MH 32-6D with strains 170-6D (mit 170), 140-8D (mit 140) and Cs 990-01B (mit 990) provided by Dr. Fukuhara. Details on statistical errors and on calculations of correction of the % loss at each dose for the spontaneous loss of markers will be published elsewhere (M. Heude and E. Moustacchi, in preparation). Figures in brackets correspond to complete ρ^- and the upper figures to sectored plus complete clones.

to a reduction of the genetic information in the ρ^- after LH.

For exponential phase cells (Table III), at low doses of UV a reduction in the frequency of marker loss is seen on immediate plating. This is followed by a progressive increase but the frequencies do not always exceed the spontaneous loss. LH reduces the loss probability for untreated cells and has either no effect or favors the rescue of certain markers (O^R_{II} and mit 140). This is probably related to the arrest in degradation of mDNA (Table II).

These experiments also show that the loss of individual markers is unequal (Table III). As after other treatments (10) the O^R_{II} and mit 140 segment is lost, even spontaneously, with a high probability.

ρ^- mutants result from deletions of the mDNA followed by repetitions of the non-deleted sequences; moreover the formation of hybrid molecules by recombination between different ρ^- has been demonstrated (for review, see 11). These facts allow us to suggest that recombinational events are likely to take place after limited mDNA degradation in irradiated growing cells. This process appears to be inducible since a) the dose response has a complex pattern (Table III) b) splitting of the UV dose leads to an increase in ρ^- and in marker loss which is abolished by cycloheximide (E. Moustacchi and M. Heude, in preparation).

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RECOMBINATION AND REPAIR IN SIMPLE EUKARYOTES

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It has been known for some time that treating cells with mutagens like ultraviolet light or ionising radiation causes recombination [1, 2, 3]. That the treatment is causative rather than selective can be demonstrated in the usual way by observing an absolute increase in numbers of recombinants per cell plated after mild levels of treatment.

The idea that recombination may be a repair mechanism has old roots. It may have been in the mind of Luria[4] following his observation of multiplicity reactivation in bacteriophages. It was enlivened by the discovery by Clarke and Margulies[5] that recombination-deficient mutants of *E. coli*, of which they isolated alleles at three loci, were extremely sensitive to both ultraviolet light and to X-rays. In *Ustilago maydis*, Holliday, who took the converse line of reasoning - that repair may be part of the recombination mechanism - also isolated radiation-sensitive mutants and found that two of them blocked UV-induced recombination. One of these turned out also to be a meiotic lethal. Both, like the bacterial mutants were also X-ray sensitive. Holliday's inspired theory of recombination in fungi has stimulated searches for similar mutants in other eukaryotes. Snow isolated several UV-sensitive mutants in *Saccharomyces cerevisiae*[8] and tested them for UV-induced gene conversion[9]. Similarly, Resnick[10] and Game and Mortimer[11] have isolated mutants of *Saccharomyces* sensitive to both X-rays and UV. Similar mutants have been found in a number of simple eukaryotes including *Neurospora crassa*[12], *Schizosaccharomyces pombe*[13], *Aspergillus nidulans*[14] and *Chlamydomonas*[15].

In *E. coli*, Howard-Flanders and his colleagues demonstrated that the repair of ultraviolet light damage was a double mechanism[16]. Most damage is repaired by excision repair, a pathway controlled by the "uvr" genes.

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Pyrimidine dimers which are unexcised generate single-strand gaps and it is the repair of these gaps which is controlled by the "rec" genes. Rupp et al.[17] showed that this repair involved the exchange of DNA strands between sibling molecules and termed this activity 'recombination repair'. This scheme has since been taken as the model for repair in eukaryotes and the fact that many of the mutant phenotypes have obvious similarities to those of the bacterial "rec" mutants has been taken as an indication that repair by recombination operates in eukaryotes too.

What is the evidence that it does? First, it has to be admitted that there has, in eukaryotes, been no direct biochemical demonstration of the exchange of parts of homologous DNA molecules in the course of repair. However, in spite of this, there is some evidence, which in some ways is even more telling. Let us start with the most circumstantial and move towards the direct.

First, there is the evidence offered by the properties of pleiotropic radiation-sensitive mutants. Clearly it is not very informative merely to show that a mutant is sensitive to both ultraviolet light and to ionising radiation; and not much more so if it is a meiotic lethal. However, many radiation-sensitive mutants alter the frequency of radiation-induced mitotic recombination[7, 9, 12, 14], when the rates are compared with those in wild-type over a similar range of doses. If it were possible to correlate these properties with the functions of the mutants in repair pathways determined by other methods, it would constitute a powerful indication that particular repair pathways involved recombination. In bacteria and in yeast, it has been possible to make this deduction rather convincingly about the role of repair in radiation-induced mutagenesis[18,19]. When a similar comparison is made of recombination among the mutants of yeast, the results are rather equivocal. Table 1 lists the effects of some mutants on recombination induced by UV and X-rays. There are two points to be made. First, this data includes no mutant in which all mitotic recombination is blocked. There is unpublished data (S. Nakai, personal communication; M. Resnick, personal communication) indicating that in the X-ray sensitive mutants, rad51, rad52 and rad54, neither intra-

genic recombination nor intergenic recombination is induced by either UV or X-rays. However the mutants of Table 1 represent each of the three UV repair pathways demonstrated by Game and Cox[19]. Blockages in any of the three pathways appear to enhance intergenic recombination. The effect on intragenic recombination is similar, but less marked. This may indicate one of two things. Either, we may expect to find that recombination induced by UV light is irrelevant to the survival of the cell: that is that there is a perfectly straightforward series of metabolic events induced by radiation leading to recombination, but not contributing to repair at all. Alternatively it may be that all pathways are somewhat recombination-prone and the rates of recombination observed in the various mutants are the outcome of the relative contributions they make to repair and recombination when wild-type. This seems a possible explanation of the intragenic recombination results, where there is some variation between 'pathways', but not of the measurements of intergenic recombination, where all mutants have similar quantitative effects.

Secondly, it is noticeable that, following X-ray treatment, the effects of mutants on intragenic recombination and on intergenic recombination may be quite different. (Compare rad6 and rad15). Discrepancies in the responses of intra- and inter-genic events to radiation and to post-treatment have been noted previously[20]. Nakai (personal communication) has obtained copious data on the effects of various mutants on radiation and induced recombination in yeast and has come to the conclusion that radiation induced mutation, intragenic and intergenic recombination are subject to separate but overlapping genetic controls. These may be summarised as follows:

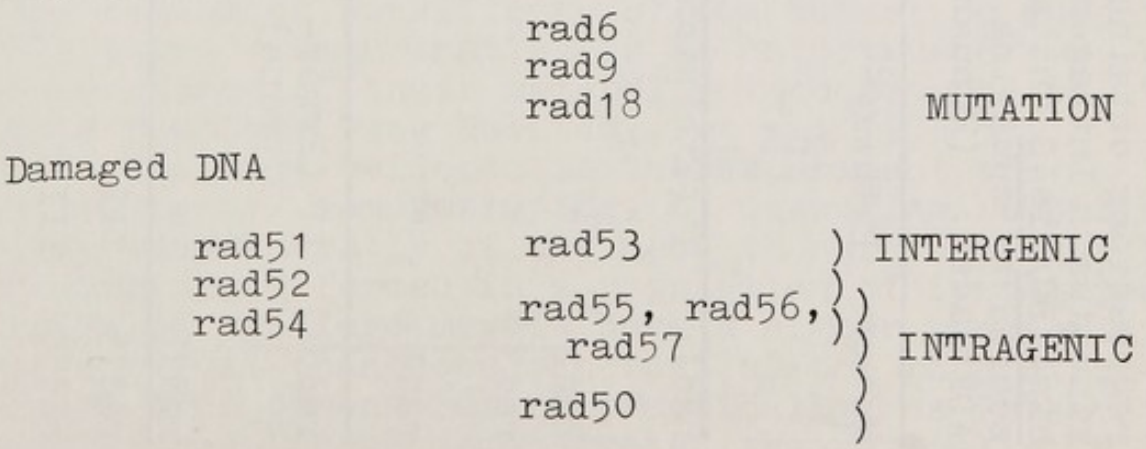

TABLE I The ratios of the rate of recombination in rad/rad compared to that in RAD/rad (wild-type) measured form log-log plots where, except for *, the regressions obtained were all approximately indicating dose square induction kinetics. Intragenic recombination rates were scored as prototrophy at the his4ABC/his4C and at the trp5U9/trp5U6 loci. Reciprocal recombination rates were scored by counting red colonies in the same strains, all being heterozygous ADE+/ade2. (Hunnable and Cox, Mutation Res. 13, 297 (1971)).

Mutant	Pathway[20]	Meiosis	Ultraviolet		X-rays	
			Intra-genic	Inter-genic	Intra-genic	Inter-genic
rad1	I (Excision)		2.2	-	1.0	1.0
		Normal	3.6			
rad2	"	"	14 - 16	26		
rad3	"	"	3.0 - 4.6	27		
rad4	"	"	3.0 - 9.0	12		
rad6	II (Error-prone)	Not completed	2.2	12	2.7 - 6.2	0*
rad18	"	Normal	2.2		2.7	2.3
rad15	III	Normal	1.0		0.31	2.3
rad50	III	Lethal	1.0 - 1.9	10	0.30	

This scheme correlates with the presumed pathways of repair of X-ray damage in yeast based on survival data from double mutants (ref. 11 and Nakai, unpublished results). While this implicates such repair pathways in recombination, or in mutation, it does no more than suggest that they may be error-prone or rec-prone, not that recombination is the means by which repair is effected.

The next evidence is from some very elegant experiments by Brünborg and Williamson using synchronised cultures of yeast in which they demonstrate that a period of high resistance of yeast to X-rays is confined to S and G2 and terminated abruptly by mitosis. The coincidence of the resistance with the presence in the cell of sibling chromatids strongly implies a requirement for the ability to recombine in the course of repairing the damage.

Next, Resnick[22] and Ho and Mortimer[23, 24] have shown that this resistance is due to the ability of yeast to repair dominant lesions which are double strand breaks. Briefly, the observations are that the repair of dominant lesions, scored by Mortimer's mating assay and of double-strand breaks, assayed in neutral sucrose gradients are both controlled by the same gene, rad52. Resnick[25] has further shown that there is a one to one quantitative relationship between the induction of double-strand breaks and of intragenic recombination by X-rays per unit length of DNA, implying that the repair of one leads to the other.

None of these evidences rule out the possibility that recombination is either incidental to repair and not a necessary part of it, as may be the case with double strand break repair; or that recombination follows the gratuitous induction of recombination enzymes by the mutagenic agent and the radiation sensitivity of the mutant or mutants 'blocking' recombination is an unfortunate consequence of their metabolic incompetence in this reaction (see Holliday, et al. 1976[26]).

The last evidence is the experiment of Holliday's[28] demonstrating, by measuring recombination independently of the need to survive, that the recombinants formed in a population of irradiated cells of Ustilago maydis have a higher probability of survival than does the rest of the population. Apart from demonstrating practically, as Eckhardt and Haynes[29] have demonstrated theoretically, that

much of the discussion of revolving around the significance of linear and dose-squared kinetics of induction of genetic changes has been based on erroneous assumptions, Holliday's data leave little doubt that recombination improves the survival chances of irradiated cells.

Finally, a sense in which recombination contributes to repair has escaped the attention of most people except P. Unrau[30]. He has published evidence that in Ustilago, an organism which spends stationary phase and much of its cell cycle in G2, recombination appears to improve the ability of the cells to excise dimers, presumably by separating dimers which are too close together and liable to interfere with each other's repair[31] into separate chromatids. It is worth considering that in the majority of eukaryotes, which are diploid, this could be an important role of recombination, making a significant contribution to the ability of cells to survive damage to their DNA.

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ULTRAVIOLET LIGHT INDUCED MUTAGENESIS IN *SACCHAROMYCES CEREVISIAE*[1]

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ABSTRACT UV-induced mutations in *Saccharomyces* appear to arise from the action of a multi-step, branching process, different branches giving rise to mutations of different kinds or at different genetic sites. Mutations may occur at some distance from the causal photoproduct, and almost always involve single base pair changes. These observations are difficult to explain according to a single-step unitary model of the kind proposed by Radman (2), and may indicate that prokaryotes and eukaryotes differ in this respect.

Genetic analysis of UV mutagenesis in the yeast *Saccharomyces Cerevisiae*, making use of radiation sensitive mutants deficient in this process and the reversion of well defined mutations in the structural gene for iso-1-cytochrome *c* suggests that mutations are induced in DNA as a result of the joint action of a number of gene products and that different base pair changes, or mutations at different genetic sites, are produced by partially different sets of gene functions (1). Such a multi-step branching model contrasts with the single-step unitary model proposed by Radman (2), and developed by Witkin (3), for bacterial mutagenesis and may imply that prokaryotes and eukaryotes differ in this respect. The yeast data also show that mutations can arise at a distance from the causal photoproduct and the work of Sherman and Stewart (see 1 for references) demonstrates that the great majority of these mutations are the consequence of a single base-pair alteration. Such a low frequency of double base-pair mutations also appears to be inconsistent with the bacterial model.

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Genes Involved in UV Mutagenesis Have Variable Mutational Phenotypes. Mutations at the *REV*1, *REV*2 and *REV*3 loci, isolated by Lemontt (4) from strains deficient in UV-induced reversion of the ochre allele *arg*4-17, have very diverse effects on the reversion of a set of well defined, contrasting, *cyc*1 alleles. With the possible exception of one incompletely understood example, the *REV*1 gene function is not concerned with base pair additions or deletions, and although required for the production of base-pair substitutions of many kinds at many genetic sites, it is also not required to produce the G·C to A·T transitions that revert the proline missense allele *cyc*1-115 and the initiation mutant *cyc*1-131 (1). The *REV*1 function is required, however, to produce the G·C to A·T transitions which revert the proline missense mutant *cyc*1-6, and also to produce the transversion which reverts the initiation mutant *cyc*1-51 that is altered at an identical base-pair site to *cyc*1-131. Since photoreactivation removes a high proportion of the induced revertants of *cyc*1-115 and *cyc*1-131, and also of all other *cyc*1 alleles examined, both in excision proficient and deficient strains exposed to low fluences where the yield of revertants is linearly related to fluence, there is no evidence to indicate that *cyc*1-115 and *cyc*1-131 revert as a consequence of an unusual premutational lesion (1).

The *REV*2 function appears to be concerned exclusively with the reversion of some, but not all, ochre alleles, and acts to enhance their reversion frequency (5). Since the yield of induced revertants of *arg*4-17, one of the ochre mutants in this category, increases in proportion to the square of the fluence in *REV*2+ strains, but is directly proportional to fluence in *rev*2-1 diploids, the *REV*2 gene product appears to facilitate the conversion of two otherwise inactive photoproducts into a single premutational lesion (5).

The *REV*3 gene function is required for the induction of nearly all base-pair substitutions, additions and deletions, except for about half of the substitutions that revert *cyc*1-115 and *cyc*1-131 (6). These alleles cannot be reverted by UV in strains carrying *rad*6-1, and the *RAD*6 gene product appears to be essential for mutagenesis of all kinds and at all sites (6, 7).

Different Genetic Sites Exhibit Characteristic Types and Frequencies of Mutation. Each of the *cyc*1 alleles that have been studied exhibit characteristic UV-induced reversion frequencies in wild type strains, and these may differ by two to three orders of magnitude (1, 5). Moreover, ochre mutations located at different sites within the *cyc*1 gene revert by means of characteristic sets of substitutions (8) as well as at particular frequencies.

Most of this variation appears to be the consequence of site specific interactions of repair enzymes with DNA, rather than variable detection or recovery of mutations, or non-random interaction of the mutagen with the genetic material. Variable recovery of mutations could arise from varying functional restrictions at different genetic sites or from redundancy in the genetic code, but such factors account for very little of the variation in the data under consideration (6). UV interacts non-randomly with DNA, depending largely on the number and type of adjacent pyrimidines, but this too appears to account for only a small amount of the variation in mutation frequency. This frequency is not correlated in any way with the composition of the nucleotide sequence consisting of the site of mutation together with the two flanking base pairs, and the frequency can vary by almost a factor of a hundred within a single defined sequence (6). This is not surprising, since mutations can arise at some distance from the causal photoproduct; evidence given in the previous section indicates that *cyc*1-131 reverts as a consequence of cyclobutyl pyrimidine dimers, but the site of the reversion lies within a sequence of alternating purines and pyrimidines, that is at a site where dimers cannot form (1). Since the reversion frequency of *cyc*1-131 is very similar to those of other initiation mutants that do not share this feature, there is no reason to believe that "mutation at a distance" is a rare event. Alleles that revert very well with UV tend to lie in regions rich in potential dimer sites, but the significance of the small sample of mutations of this kind is difficult to assess and other alleles that occur in similar regions often revert poorly; at best, an abundance of adjacent pyrimidines could be a necessary, but not a sufficient condition for a high reversion frequency.

Comparisons between the ratios of reversion frequencies of various alleles in excision proficient and deficient strains (1) suggests that part of the variation in reversion frequency of those alleles is due to varying amounts of mutagenic repair relative to non-mutagenic repair at different genetic sites, but a considerable difference between alleles still exists in strains lacking excision. Such an explanation is also inadequate to explain variation in the types of base pair change at different sites. Although there are no functional restrictions, 90% of the UV-induced reversions of *cyc*1-9 involve an A·T to G C transition at the first position of the ochre codon, a substitution which is much less common among the revertants of ochre mutants located at other sites (8). Such specificity depends on the action of the *RAD*6 gene (9). It seems likely, therefore, that the non-randomness in mutagenesis at different genetic sites is largely due to the non-random action of the gene products involved in this

process.

Most Mutations Are the Result of Single Base-Pair Changes. The work of Sherman and Stewart (see 1 for references) has shown that the great majority of UV-induced mutations are the consequence of a single base-pair change. Among 123 UV-induced revertants of *cyc*1-9, only one was due to two adjacent substitutions. No double base-pair changes were found in an excision defective strain, where most revertants must be caused by dimers. A low frequency of this kind is difficult to reconcile with Radman's model which predicts a majority of double base pair changes (2), although some single base pair substitutions might be expected if the editing function suppressor remains associated with the polymerase, due to the failure to correct spontaneous errors. The frequency of such errors would have to be unreasonably high to explain the yeast data, however. If such a polymerase were to fill a gap of a thousand bases, and the probability of inserting incorrect bases opposite the dimerised pyrimidines was close to 100%, the frequency of spontaneous error would have to approach 10% to explain the yeast results. Even though some of these mutations might fail to be detected because of functional restrictions, or redundancy of the code, several should be found in a gene as small as the *cyc*1 locus.

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REGULATION BY PROTEINASES OF A PUTATIVE REC-NUCLEASE OF *NEUROSPORA*[1]

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ABSTRACT Three mutants of *Neurospora* which are sensitive to ultraviolet light and nitrosoguanidine (*nuh-4*, *uvs-3* and *uvs-6*), and two mutants which are insensitive to these mutagens (*nuh-1* and *nuh-3*), have reduced levels of *expressed* endo-exonuclease activity in extracts, but wild-type levels of *total* activity, determined after activation either by endogenous proteinases or with trypsin. The *uvs-3* and *uvs-6* mutants were shown previously to have altered mitotic recombination. The expression of endo-exonuclease activity in extracts of mycelia is apparently regulated by a proteinase-sensitive "inhibitor" which preferentially suppresses the exonuclease activity of the enzyme and the levels of PMSF-sensitive proteinase(s) which convert an inactive precursor form of the enzyme into the active form. The active enzyme is a single polypeptide (53,000 daltons) with both exonuclease and single-strand specific endonuclease activities. It degrades linear but not circular double-strand DNA and both activities are inhibited by ATP. The precursor appears to be a polypeptide of 85-93,000 daltons in molecular weight.

INTRODUCTION

The single-strand (ss) specific endonuclease of mycelia of *Neurospora crassa* originally purified from stationary starved cells by Linn and Lehman (1) was found previously to co-purify from log phase cells with a double-strand (ds) exonuclease activity (2). Together these activities degraded linear but not circular ds-DNA and both were inhibited 0.1-1.0 mM ATP. These properties and the ability of the ss-endonuclease to sense ultraviolet light (UV) damage in ds-DNA suggested a possible involvement in DNA-repair and recombination (3). The ds-exonuclease activity was preferentially inactivated in the presence of high salt, by treatment with EDTA and on "ageing". The latter effect was retarded by added protein and thus seemed likely to be the result of proteolysis. At least two distinct proteinase activities were detected in the purified preparations. It was suggested (2) that the ss-endonuclease was an end-product of proteolytic

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"processing" of what is now called (see below) endo-exonuclease. Whether this processing occurs *in vivo* in starved cells or is an "artifact" of extraction and purification is still not clear. Mutants of *Neurospora* have now been obtained which affect the intracellular levels of endo-nuclease and further implicate an involvement of the enzyme in DNA-repair and recombination. Additional properties of the endo-exonuclease have been found which suggest that proteinase(s) regulate the expression of this enzyme *in vivo*.

RESULTS AND DISCUSSION

Mutants. Nine new mutants of *Neurospora* have been isolated, characterized and mapped (Fraser and Käfer, unpublished) which all failed to release normal amounts of DNase activity with properties in common with endo-exonuclease when the mutants were grown in colonial form on DNA-agar. The release of DNase was recognized by reduced haloes of digestion around the mutant colonies as compared with wild-type after the undigested DNA was precipitated with acid. The mutants are thus called nuclease halo or *nuh* mutants. Three *nuh* mutants (*nuh-1* *nuh-3* and *nuh-4*) and two UV-sensitive mutants which also had the Nuh phenotype (*uvs-3* and *uvs-6*) showed reduced levels of *expressed* endo-exonuclease activity (Table 1). The *nuh-4* mutant proved to be sensitive to both UV and nitrosoguanidine (NG) (Käfer and Fraser, unpublished) as found previously for *uvs-3* and *uvs-6*, two mutants that also have high mitotic recombination (4). The *total* levels of endo-exonuclease in extracts were revealed after treatment with trypsin

TABLE I
RELATIVE SPECIFIC ENDO-EXONUCLEASE ACTIVITIES[a]
IN MUTANTS OF *NEUROSPORA*

Strain	Expressed levels (%)		Total levels (%)	
	MID-LOG	END-LOG	MID-LOG	END-LOG
Wild-type	100	100	100	100
nuh-1	28	65	60	69
nuh-3	39	40	81	100
nuh-4	67	60	90	99
uvs-3	33	40	90	86
uvs-6	67	80	86	88

[a]Measured as ss-DNase activity in extracts of log phase mycelia before (*expressed* levels) and after (*total* levels) trypsin activation. These values are averages of 3-8 separate determinations which agreed within 10-15% of the value shown.

(see below). Only *nuh-1* was significantly deficient in *total* enzyme. A co-ordinate reduction in the release of proteinase activity (pepstatin-sensitive caseinolytic activity at pH 8.0) was also observed in preliminary experiments for all of the mutants shown here except *nuh-3*.

Expression of Endo-exonuclease Activity in Extracts of Wild-type. The ss-DNase and ds-DNase activities in extracts of wild-type *Neurospora* were activated respectively 3.8- and 21.0- fold on standing at room temperature (Table II). The activations were prevented by the proteinase inhibitor PMSF and occurred to the same levels when fresh extracts were treated with trypsin indicating that proteinase(s) were "unmasking" an inactive form of endo-exonuclease. After extracts were passed through DEAE-cellulose, the activation of both ss-DNase and ds-DNase activities by endogenous proteinase(s) or with trypsin still occurred, but both were activated to the *same* extent. It thus appears that there is a proteinase-sensitive "inhibitor" in crude extracts which preferentially suppresses the ds-exonuclease activity of the endo-exonuclease. The "inhibitor" has not been positively identified as yet. The inactive form of the endo-exonuclease appears to be a precursor form of the enzyme (see below). Both the precursor and the ds-DNase "inhibitor" were found to accumulate during the log phase growth and to disappear when the mycelia entered stationary phase.

Purification of Active and Inactive Forms of Endo-exonuclease. When partially purified preparations of active endo-exonuclease (2) were run in 6 M urea-polyacrylamide gels, active enzyme was recovered as a single fast-running component, an acidic protein. On SDS-gels this proved to be a single polypeptide, but the molecular weights of the polypeptide recovered from different preparations varied from 33,000 to an upper limit of about 53,000 suggesting that active fragments had undergone different extents of proteolysis.

TABLE II
PMSF[a]-SENSITIVE ACTIVATION OF ENDO-EXONUCLEASE IN EXTRACTS OF MYCELIA

Time at room temperature (days)	Nuclease activity (units/ml) ss-DNase -PMSF	ss-DNase +PMSF	ds-DNase -PMSF	ds-DNase +PMSF	Ratio (-PMSF) ss-DNase / ds-DNase
0	88	91	3.0	2.2	29
1	338	103	63.0	2.2	5.3

[a]Phenylmethylsulfonyl fluoride, a proteinase inhibitor.

TABLE III
PURIFICATION[a] OF ENDO-EXONUCLEASE PRECURSOR FROM EXTRACTS OF LOG-PHASE WILD-TYPE MYCELIA

Fraction	Total Protein (mg)	Trypsin-activatable DNase ss-DNase (total units)	Trypsin-activatable DNase ds-DNase (total units)	ss-DNase Recovery (%)	ss-DNase PFN (-fold)
Extract	588	10,200	2,600	100	1.0
DEAE	188	5,740	1,270	56	1.8
OS	26	2,210	255	22	4.9
HAP	1.9	1,560	225	15	47

[a]Three steps in purification were used: chromatography on DEAE-cellulose, octyl-Sepharose (OS) and hydroxyapatite (HAP).

The inactive precursor was isolated free of active enzyme (hydroxyapatite fraction, Table III) after three chromatographic steps. It was assayed at each stage after activation with trypsin. A trypsin-sensitive polypeptide of molecular weight 85-93,000 daltons has been identified which most probably corresponds to the precursor form of endo-exonuclease. It was also recovered from 6 M urea-polyarylamide gels, but the specific activity after trypsin activation was low, perhaps because of denaturation. The precursor is a relatively hydrophobic protein since it bound strongly to octyl-Sepharose and phenyl-Sepharose, a property in common with other precursor enzymes and proteins which are secreted from cells.

The results indicate that the expression of endo-exonuclease in *Neurospora* may be regulated by proteinase(s) which convert the precursor to the active enzyme and which destroy the ds-exonuclease "inhibitor". The reduced expression of endo-exonuclease activity in mutagen-sensitive mutants indirectly implicates this enzyme in DNA-repair and possibly also in recombination.

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GENETICS OF DNA REPAIR IN THE CELLULAR SLIME MOLD, DICTYOSTELIUM DISCOIDEUM[1]

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ABSTRACT Parasexual genetic analyses of radiation-sensitive mutations of Dictyostelium discoideum indicate that they fall into at least nine complementation groups located on several of the seven genetic linkage groups. Further studies using double mutant haploids show that these mutations affect at least two repair pathways one of which appears to be excision repair.

INTRODUCTION

The cellular slime mold, Dictyostelium discoideum, which has been used in studies of microbial differentiation and development (1,2), is also a promising eukaryotic system for studying DNA repair (3-6). Mutants sensitive to ultraviolet light (UV;254 nm), ^{60}Co gamma rays, and chemical mutagens can be readily isolated (3,7). Studies aimed at elucidating the repair deficiencies of these mutants have been initiated (4,6,8). The genetic characterization of these mutations is basic to further investigations of repair in this organism.

RESULTS AND DISCUSSION

Genetic analyses of mutants of D. discoideum, including those which are radiation-sensitive, have made use of the parasexual cycle of this microorganism (7,9-12). Haploid strains bearing the radiation-sensitive mutations are allowed to fuse spontaneously with other haploid strains of the same mating type which bear mutations that serve as genetic markers for the seven linkage groups (chromosomes) of D. discoideum. The diploid strains which result from such fusion events are most commonly selectively isolated as temperature-resistant members of mixed populations of the temperature-sensitive haploid parental strains at frequencies of about 10^{-5} (9-11). Cells in the diploid population then

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lose chromosomes, possibly through a process of transient aneuploidy, to form segregant haploids which are selectively isolated from the diploid population at frequencies of about 10^{-4} using recessive drug-resistance markers (10,11). The chromosomes of the initial parental haploids assort independently into the segregant haploids, allowing the linkage of the radiation-sensitive mutations to be determined from the types of segregants produced. For example, when a radiation-sensitive (radA20), cycloheximide-sensitive haploid is fused with a radiation-resistant, cycloheximide-resistant (cycA1) haploid the segregant haploids obtained are either radiation-sensitive, cycloheximide-sensitive or radiation-resistant, cycloheximide-resistant (12). No radiation-resistant, cycloheximide-sensitive or radiation-sensitive, cycloheximide-resistant haploids are obtained. Therefore the radA20 mutation is on the same linkage group as the cycA1 mutation.

The complementation of the radiation-sensitive mutations can also be studied in diploids formed by fusion of pairs of haploids each of which bears a different radiation-sensitive mutation. All the radiation-sensitive mutations so far studied are recessive since diploids formed by the fusion of a radiation-sensitive haploid with a haploid having a wild type radiation resistance have the same UV sensitivity as diploids formed by fusion of two radiation-resistant haploids (7,12). The UV sensitivity of these diploids differs only slightly from that of the wild type haploid (7,12). Mutations were judged to be complementary and therefore to affect different genes if the diploid bearing both mutations had a UV sensitivity like that of the diploids formed by fusion of two radiation-resistant haploids. Mutations were judged to affect the same gene when the diploid had a UV sensitivity similar to that of the parental radiation-sensitive haploids.

The results of linkage and complementation tests with some of the currently available radiation-sensitive strains indicate that mutations in at least nine loci affect the repair of DNA in D. discoideum following irradiation with UV or ^{60}Co gamma rays (7). Eight of these loci have been assigned to particular linkage groups as shown in Table 1. The linkage of the radE mutation has not been established; however, complementation tests indicate that this mutation is not allelic with any of the other radiation-sensitive mutations studied to date (Dr. M. B. Coukell, personal communication; Welker and Deering, unpublished data). Two independently isolated mutations that mapped on linkage group I, radA20 and radA22, were assigned to the radA locus on the basis of the non-complementarity of these mutations in a radA20/radA22 diploid. The radC35 and radC44 mutations were also assigned to a single locus on the basis of their non-complementarity. Similar complementation tests with the radD14 and radF21

TABLE I
LINKAGE OF RADIATION-SENSITIVE MUTATIONS OF D. DISCOIDEUM

Linkage group	Markers: Radiation sensitive[a]	Markers: Other[b]
I	radA	acrB, aggB, cadA, cycA, devA, sprA, tsgE, tsgI
II	radH	acrA, aggA, aggF, aggI, axeA, devB, tsgD, tsgF, tsgH, whiA, sprB
III	radC	acrC, axeB, tsgA
IV	radD, radF	aggJ, bwnA, ebrA, tsgB
V	radG, radI	bsgA[c], tsgC
VI	radB	manA
VII		cobA
(?)	radE[d]	

[a] Linkage determined in this laboratory.
[b] Adapted from Newell, Ratner and Wright (13).
[c] Recent experiments suggest that bsgA (and thus radG and radI) may instead be on linkage group III (D. Ratner and P. C. Newell, personal communication).
[d] Linkage unknown at present.

mutations on linkage group IV and also the radG5 and radI24 mutations on linkage group V indicated that these mutations affect different loci.

The radC mutations are thought to affect a UV-specific endonuclease since few single-strand breaks are produced in the nuclear DNA of radC strains compared to their parental strains following a 15 J/m^2 UV irradiation (8). In addition the radC mutations increase UV sensitivity but not ^{60}Co gamma ray sensitivity (7). A strain bearing the radC35 mutation had a higher UV-induced mutation frequency than its parental strain, in analogy with excision-defective strains of yeast and Escherichia coli (14-16). Thus the radC mutations probably affect an endonuclease involved in the excision repair of UV-induced DNA damage.

The interactions of some of the radiation-sensitive mutations in double mutant haploids have also been studied (17). Double mutant haploids bearing both the radA20 and radC44 mutations were more sensitive to UV irradiation than either single mutant haploid, indicating that the radA and radC gene products are involved in different repair pathways. Double mutant haploids carrying the radA20 and radB13 mutations had the same sensitivity to UV and ^{60}Co gamma rays as the radB13 single mutant, indicating that the radA and radB gene products

are involved in the same repair pathway. If the radC mutations affect an excision repair pathway, then the radA and radB gene products may be involved in some other type of repair, possibly some form of postreplication repair.

It is now possible to construct new radiation-sensitive strains to meet the needs of the experimenter. In particular, radiation-sensitive strains able to grow on a defined medium have been constructed and used to study the synthesis of DNA following UV irradiation (J. Kielman, personal communication).

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DNA REPAIR IN *DROSOPHILA*[1]

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ABSTRACT. Five genes which influence DNA repair have been identified by analysis of mutagen sensitive strains of *Drosophila melanogaster*. The *mei-9* mutants are deficient in the excision of pyrimidine dimers. Reduced postreplication repair has been associated with four other complementation groups.

INTRODUCTION

Drosophila has recently been employed as a model for the analysis of DNA repair in multicellular eukaryotes, because it permits a combined application of powerful genetic, cytogenetic, and biochemical approaches (1). Over 60 years of intense genetic experimentation has provided the necessary tools for selection and characterization of mutants potentially deficient in DNA repair (2). Refined procedures are also available for the analysis of chromosome stability, exchange, and segregation in both germ line and somatic cells. Finally, advances in insect tissue culture have made it possible to analyze each of the major known forms of DNA repair using procedures developed with mammalian cells (3, 4).

MUTANTS

Studies of strain sensitivity have implicated several Drosophila mutants in DNA repair (5, 6, 7, 8). The first systematic search for repair-deficient mutants was reported by Smith, who selected for hypersensitivity to methyl methanesulfonate (9). That group, together with several others have since employed similar procedures to recover mutants which now define over 20 complementation groups (10, 11, 12, 13, 14, 15, 16). A partial overlap was soon discovered between the mutagen-sensitive strains, termed *mus*, and mutants which had been recovered in a search for deficient meiotic functions, labeled *mei* (17). This redundancy implies that an analysis of DNA repair in mutants common to both classes may also reveal something of the mechanism of meiotic recombination.

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PHOTOREPAIR

Direct and indirect analyses of pyrimidine dimers have revealed an efficient photorepair system in Drosophila tissue culture cells (18, 4). Studies of repair replication have also indirectly detected photorepair in larvae (19). Mutants affecting this process have not yet been identified.

EXCISION REPAIR

Observations of unscheduled DNA synthesis in Drosophila polytene chromosomes following x-ray treatment suggested the existence of excision repair in this organism (20, 21). Subsequent analyses of pyrimidine dimers and repair replication firmly established its presence (18, 19). The mei-9 mutants, which have been the object of extensive genetic and biochemical analyses (22, 4), are deficient in the excision of pyrimidine dimers (Table). These mutants are distinguished from excision-deficient mutants of other organisms by their sensitivity to a broad spectrum of mutagens (Table). Meiotic recombination is reduced by 90% in females homozygous for mei-9 mutants, whereas recombination and chromosome breakage are increased in somatic cells of both sexes (17, 1). These mutants are deficient in repair replication following both uv and x-ray exposure (23). Detection of single strand breaks in mei-9 cells treated with N-acetoxy-acetylaminofluorene suggests that the block in this pathway may occur subsequent to the incision step of repair (24).

POSTREPLICATION REPAIR

Mutants from four complementation groups have been associated with reduced postreplication repair following uv exposure (Table). Two of these mutants also reduce meiotic recombination. The mei-41 mutants exhibit an exceptionally strong deficiency as well as a high sensitivity to all tested mutagens. These mutants are also associated with a dramatic increase in the frequency of spontaneous somatic chromosome breakage (1). The residual postreplication repair in the mus(1)104 mutant is more sensitive to caffeine than is that of the other mutants (4).

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TABLE

Properties of Drosophila Repair-Deficient Mutants

	Mutant Locus				
	mei-9	mei-41	mus(1)104	mus(1)101	mus(3)302
Repair capacity (3, 4)					
Excision	-				
Postreplication	+	-	-	-	-
Single Strand Breaks	+	+	+	+	+
Meiotic Recombination (16, 17)	-	-	+	-	
Chromosome Stability (1)	-	-			
Map Position (22, 25)	1-5	1-53.8	1-53	1-44.8	
Sensitivity (10, 13, 16)					
methyl methanesulfonate	+	+	+	+	+
x-rays	+	+	+	+	
uv	+	+			
nitrogen mustard	+	+	+	+	+
N-acetoxy-acetylaminofluorene	+	+	+	-	
benzo(a)pyrene	+	+	+	-	
4-nitroquinoline-1-oxide	+	+	+	-	
N-methyl-N'-nitro-N-nitrosoguanidine	+	+	+	-	

+ exhibits property.
- does not exhibit property.
blank--not tested or in question.

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Mutagen Sensitivity of *Drosophila melanogaster* IV. Interactions of X Chromosome Mutants[1]

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ABSTRACT Sensitivity to MMS has been used to isolate mutants of *Drosophila melanogaster* with potential defects in DNA repair processes. Eleven X-linked genes have been identified which control mutagen sensitivity. Patterns of sensitivity and interactions of double mutant strains suggest several pathways for DNA repair in this complex multicellular animal.

INTRODUCTION

Sensitivity to mutagenic agents has been used in viral, bacterial and fungal systems to isolate mutants which express defects in DNA repair processes. Genetic and biochemical analyses of these mutant strains have indicated that the repair processes are controlled by numerous genetic loci functioning through multiple metabolic pathways. Evidence of the applicability of this technique to *Drosophila melanogaster* (1) suggested that this organism might provide a suitable system for the study of the genetic control of these processes in animal cells. An array of mutagen-sensitive mutants have been selected (2,3,4) and several of these have been shown to be defective in DNA repair (5,6,7). The present report summarizes genetic information concerning mutants located on the X chromosome and describes preliminary studies of interactions exhibited by specific combinations of a number of these mutants.

METHODS

Methods for the isolation of *Drosophila* mutants with enhanced sensitivity to mutagenic agents have focused principally on the identification of X chromosomal loci and have

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generally employed MMS as the selective agent (1,2,3). The types of mutants which could be identified with these procedures have been limited in several ways. The use of MMS as the selecting agent may preclude the identification of loci conferring sensitivity to other mutagenic agents. Certain mutagen-sensitive yeast mutants, for example, display sensitivity only to ultraviolet light and nitrogen mustard (8) and analogous *Drosophila* mutants would not have been identified. In addition, the isolation schemes require the mutagen-sensitive mutant to be viable in the absence of mutagen treatment. Mutagen-sensitive loci which play an indispensable role in normal cellular metabolism would therefore either not be identified by present methods or be represented by hypomorphic rather than amorphic mutations. These limitations in methodology must be considered when interactions of mutagen-sensitive mutants are examined to identify alternate metabolic pathways for DNA repair (8,9).

RESULTS AND DISCUSSION

Fifty-two X-linked mutants which exhibit enhanced sensitivity to chronic exposure to MMS during the developmental cycle have been induced by EMS mutagenesis of 28,341 X chromosomes. Thirteen additional MMS-sensitive mutants have been isolated from a sample of 6850 EMS-treated X chromosomes by Boyd and his associates (3). In addition, mutagen sensitivity has been determined to be a characteristic of several previously isolated X-linked meiotic mutants (2,5,7). Complementation testing, based on resistance to killing by MMS during the developmental cycle, has identified thirteen complementation groups and eleven of these have been localized to specific regions of the X chromosome (FIGURE 1).

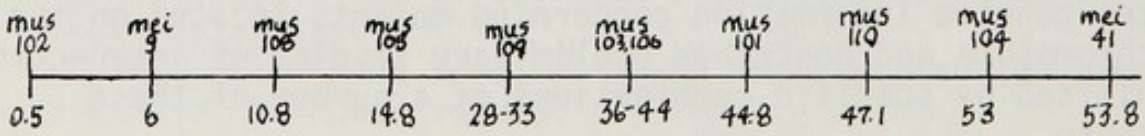

FIGURE 1. Map locations of X-linked mutagen-sensitive loci. The mutagen-sensitive loci, mei-9 and mei-41, were first identified by their effects on meiotic chromosome behavior and have retained their mei designation (10). Map locations were determined from genetic and cytogenetic studies conducted in collaboration with J. B. Boyd and J. M. Mason.

Each of the MMS-sensitive loci have been tested for sensitivity to ultraviolet light, nitrogen mustard and x-irradiation and two major patterns of sensitivity appear. One group of mutants exhibits sensitivity only to MMS and x-irradiation and includes mus 102, mus 105 and mus 106. A second

group exhibits sensitivity to all four agents tested and includes mus 101, mus 104, mus 109, mus 110, mei-9 and mei-41. The sensitivity of mus 103 appears specific for MMS.

Mutagen sensitivities exhibited by multiply-mutant combinations of radiation-sensitive strains of *Saccharomyces cerevisiae* have provided genetic evidence to support interacting pathways of DNA repair (8,9). Epistatic relationships exhibited by double mutants of rad 1,2,3,4,10 and 16 suggested defects in a common pathway of DNA repair. Genetic (11) and biochemical (12,13) evidence indicated that these loci affected excision repair. Additional double mutants which exhibited synergistic relationships with respect to radiation-induced cell killing suggested alternate methods for repair of DNA damage. A second pathway, specified by rad 6,9,18 and rev 1,2,3, appears to be necessary, for UV mutagenesis and, by analogy to bacterial systems, has been hypothesized to function as an error-prone recombinational repair pathway (8). rad 50 and 51 appear to specify a third apparently error-free pathway for repair of X-ray damage (8).

Multiple mutant studies with our X-linked MMS-sensitive mutants have detected three specific types of interactions (TABLE 1).

TABLE 1

DOUBLE MUTANT RELATIONSHIPS

	Double Mutant		Interaction
A.	mus 102	mus 105	synergistic
	mei-9	mus 105	interacts
	mei-9	mei-41	interacts
	mus 102	mei-41	interacts
B.	mus 105	mei-41	lethal
	mus 101	mei-41	lethal
C.	mus 102	mei-9	epistatic

Double mutant combinations identified in Group A exhibit an interaction between the two mutants with respect to sensitivity to killing by MMS. Of these four multiply-mutant strains, the mus 102 mus 105 interaction is clearly a synergistic one, suggesting that these two loci represent different interacting pathways. Present data do not yet allow strong conclusions concerning the strength of the interaction exhibited by the other three combinations represented in Group A.

Group B combinations are analogous to certain double mutants encountered with *E. coli*. Bacterial double mutants involving pol A and rec A, rec B, uvr E, mut U, pde B,

lig or uvr B appear to be inviable (14). Genetic data support the conclusion that the double mutants indicated in Group B cannot be constructed and, therefore, represent loci specifying gene functions which play important roles in cellular metabolism in the absence of exogenous mutagen treatment. The double mutant, mus 102 mei-9, represents a case of epistasis in which the multiply-mutant strain is no more sentitive than the more sensitive single mutant, mei-9. In view of the observation that mei-9 is defective in excision repair (6), this result suggests that mus 102 may be defective in an excision function. Though clearly preliminary, these double mutant experiments suggest the possibility that this large array of MMS-sensitive mutants may be organized to define interacting pathways of DNA repair in a complex multicellular animal system.

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WORKSHOP SUMMARY: DNA STRAND BREAK REPAIR IN EUKARYOTES[1]

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Ionizing radiations and a number of chemical agents produce strand breaks in DNA. It is well established that many such strand breaks are rapidly repaired. However, far less is known about the repair of such lesions than, for example, of pyrimidine dimers produced by the action of ultraviolet light on DNA.

One reason is the tendency for strand breaking agents to produce many types of lesions in DNA in addition to strand breaks; ionizing radiations are notorious in this respect. The major reason, however, is the relatively small number of strand breaks in cellular DNA after exposures to strand-breaking agents for which the ability of cells to form colonies has dropped substantially. This is not because strand breaks are hard to detect. Indeed, the most sensitive way to detect many lesions, including pyrimidine dimers, is to convert them to strand breaks - see the workshop summary on quantitation of damage in DNA by A. Ganesan (this volume). Instead, one infers that breaks in DNA strands have such a strong effect that only a few (compared, e.g., with pyrimidine dimers) can be tolerated per functioning cell. Therefore, progress has depended greatly on the development of sensitive enough methods for measuring strand breaks and their disappearance, or repair.

Measurement of DNA single-strand breaks. The most used method is that of alkaline gradient sedimentation (McGrath and Williams, 1966). Numbers of breaks can be determined with reasonable accuracy, when exposure to strand breaking agents (e.g. X-rays) has reduced the single-strand molecular weight to about 5×10^8 daltons or less.

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For smaller numbers of breaks (larger single-strand DNA), reliable measurements do not seem possible by alkaline sedimentation. Complications which have been observed under various conditions include: failure of the single strands to sediment as free and independent molecules (because they have structures involving proteins, lipids, supercoiled regions etc.); sedimentation anomalies of large DNA because of changing sedimentation coefficient with centrifuge rotor speed (Zimm, 1974); slow hydrolysis in alkali of the phosphodiester bonds, perhaps catalyzed by light (Elkind, 1971; Friedman, et al., 1975).

X-rays induce single-strand breaks in intracellular DNA at rates of 1 - 5 breaks per 10^9 daltons per kilorad, so that 0.4-2 kilorads are required to get the number-average molecular weight low enough for measurement by alkaline sedimentation. Thus, strand breaks may be measured at radiation exposures which halve the colony forming ability of typical bacteria (1-15 kilorads) or lower eukaryotes such as yeasts (1-20 kilorads). For mammalian cells in cultures, however, only 100 rads halves colony forming ability, so sedimentation measurements may only be made on DNA from cells which have been irradiated too heavily to be able to replicate.

Ahnström and his colleagues have introduced a technique in which the presence of strand breaks is measured by an increase in the rate at which double-helical DNA unwinds under alkaline denaturing conditions to produce single strands (Ahnström and Erixon, 1973; Ahnström and Edvardsson, 1974). Experimental results obtained using this method are in reasonable agreement with the theory of DNA unwinding (Rydberg, 1975). The method will measure changes in the amount of unwound DNA after only 100 rads to mammalian cells. However, the method is sensitive to crosslinks between strands as well as to strand breaks.

At this workshop, Rydberg and Johnson (this volume) gave a preliminary description of an ingenious modification of this method which makes possible the measurement of strand breaks in single mammalian cells after irradiation with a few hundred rads. This is a particularly important development because: (1) it makes possible a comparison of strand break repair in cells which are able to form colonies and in those which can not; (2) the formation and repair of strand breaks can be studied in various parts of the cell cycle without subjecting the cells to treatments to synchronize them.

At about the same time, Kohn and associates were developing a technique for the detection of strand breaks by measurement of the rate of elution under alkaline conditions of single-strand DNA from cells on a filter (Kohn and Ewig, 1973; Kohn, et al., 1974, 1976). The method is apparently even more sensitive than the unwinding method, with detectable changes in elution for cells given as little as 30 rads. The physical basis for the observed increases in elution rate is still obscure, which raises questions in the interpretation of the data. Besides strand breaks and crosslinks between DNA strands, the elution rate is also sensitive to DNA-protein crosslinks (Fornace and Kohn, 1976), perhaps because the proteins tend to stick to the filter.

This apparent complication in the alkaline-elution assay has been cleverly utilized to develop an assay for DNA-protein crosslinks. As described by Kohn, Ewig and Ross (this volume), the difference in the rate of alkaline elution of DNA before and after proteinase treatment is a direct measure of the number of DNA-protein crosslinks. The increase after protein digestion can be correlated with the number of crosslinks by using DNA treated with trans - Pt (II) $(NH_3)_2Cl_2$, which forms such crosslinks almost exclusively. This assay is by far the most useful assay available for DNA-protein crosslinks.

The three assays measure all strand breaks in the DNA, with a double-strand break registering as two single-strand breaks. All three assays also convert alkali-labile bonds, such as AP (apurinic or apyrimidinic) sites, to strand breaks.

The repair of single-strand breaks. Many experiments show an increase in the size of DNA strands after incubation of mammalian cells treated with strand breaking agents. This is evidence for the repair of strand breaks. Time-course studies show that most breaks produced by ionizing radiations are repaired within minutes, with a smaller component repaired over a longer time. The faster component is probably analogous to the "Type 2" repair in E. coli cells, and the slower component the "Type 3 repair" (Youngs and Smith, 1976). The repair of breaks is accompanied by insertion of new bases. Experiments with repair synthesis in mammalian cells in the presence of radioisotopically labeled bases suggests that, on the average, only a few bases are inserted per strand break repaired (Painter and Young, 1972; Fox and Fox, 1973).

In discussing repair, it is convenient to divide strand breaks into various categories.

The proportion of slowly rejoining strand breaks is increased when cells are irradiated with high LET radiation (Ritter, et al., 1976; Ahnström and Edvardsson, this volume). This is consistent with the concept that the slowly rejoining strand breaks include those in which both strands contain damage at about the same position along the double helix. This includes double-strand breaks, which will be discussed later.

The rapidly rejoining strand breaks, typically 90% of the total, are widely presumed to be repaired by the model suggested by studies on excision repair. Nucleases first excise damaged structures (and perhaps undamaged nucleotides on the same strand). Polymerases resynthesize the strand, using the complementary one as a template, and a ligase seals the break. The evidence that this is so for AP sites (alkali-labile bonds which are detected as strand breaks) is reasonable - see S. Linn's report of the workshop on the enzymology of base excision repair (this volume).

Various aspects of the repair of DNA strand breaks in cultured mammalian cells exposed to ionizing radiations were reported in the workshop by Elkind and by Ahnström and Edvardsson, and by Lett, Keng and Sun on nondividing photoreceptor cells in rabbit retina (this volume). All three presentations stressed the completeness with which strand breaks are repaired. Various experimental designs enabled the speakers to infer that strand break repair was so extensive that the number left after incubation was much below the number which could be directly measured by available techniques.

An unrepaired DNA strand break is still believed to have profound biological effects. However, the inferred extent of break repair inspired all three speakers to speculate on the significance of misrepaired breaks. The assays measure only the size of DNA strands, and do not give information on whether the base sequence at the repaired site is unaltered, or even if the wrong strands are joined together.

Some possible approaches to the study of misrepaired breaks include: (1) studies on the enzymology of strand break repair, for which there are now only a few preliminary studies (Payes, 1974; Lennartz, et al., 1975; Gaziev, et al., 1975); (2) a determination of whether the repair of strand breaks is mutagenic.

DNA double-strand breaks. In biological situations, double-strand breaks produced by coincidence between random breaks in the single strand are negligible compared to those produced by a single event - X-rays (review: Hutchinson, 1975a), restriction enzymes (review: Meselson, et al., 1972) and bleomycin (Povirk, et al., 1977).

Double-strand breaks are measured by sedimentation in neutral gradients. This method becomes extremely difficult for DNA larger than about 10^9 daltons, because the sedimentation coefficient becomes strongly dependent on centrifuge speed (Zimm, 1974; Hutchinson, and Krasin, 1977). Since X-rays produce 0.1-0.2 breaks per 10^9 daltons per kilorad in aerobic cells (Hutchinson, 1975a), this limits measurements to cells which have received at least 5-10 kilorads. Viscoelastometry (Klotz and Zimm, 1972) can measure larger DNA, but is so sensitive to the size of the largest molecule in solution that it has not yet been used successfully for measurement of number-average molecular weights.

The repair of DNA double-strand breaks was in doubt for some time, except in the unusually radiation-resistant bacterium, Micrococcus radiodurans (Kitayama and Matsuyama, 1968; Lett et al., 1970; Burrell et al., 1971). It is now reasonably well established that such repair does occur in bacteria (Hariharan and Hutchinson, 1973; Krisch et al., 1976). Repair is not found in E. coli recA$^-$ cells, or in wild type cells with only a little more than one genome per cell (Krasin and Hutchinson, 1977). Reports of no double-strand break repair in E. coli (Kaplan, 1966; Bonura et al., 1975) are probably based on cells with little more than one genome per cell. In yeast cells, also, double-strand breaks are repaired, but not in rad 52 strains (Ho, 1975; Resnick and Martin, 1976). Resnick (this volume) finds no repair of double-strand breaks when the yeast chromosomes are unable to pair homologously. These results suggest that repair of double-strand breaks is a recombinational-like event (see Resnick, 1976).

The repair of double-strand breaks in irradiated mammalian cells has been reported (Corry and Cole, 1973; Cole et al 1975; Hartwig and Handschack, 1975; Lehmann and Stevens, 1977). The major problem here is that the structure of the sedimenting DNA, especially after incubation, is generally less clearly understood than for bacteria and yeast. This is not surprising, since in these latter organisms the DNA is much smaller in size. Also, mammalian cells lose essentially all ability to replicate after a few hundred rads. Therefore,

measurements of double-strand breaks are for practical purposes on cells which have been irradiated far beyond the level at which they can form colonies.

Chromosome structure and strand break repair. The rapidly accumulating knowledge of the structure of chromosomes must be the foundation for understanding the biological effects of DNA strand breaks and of their repair in mammalian cells. It is probable that strand break studies can contribute to the knowledge of chromosome structure. For example, measurement of supercoiling of DNA in cells has depended in part on studies on the effect of strand breaks (Cook and Brazell, 1976).

The concept of chromosome aberrations as unrepaired DNA double-strand breaks has been advanced by Bender and his associates (Bender, Bedford and Mitchell, 1973). The discussions by J. German and S. Wolff (this volume) of sister chromatid exchanges bring to mind the possibility that the misrepair of strand breaks may be involved.

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ESTIMATION OF DNA STRAND BREAKS IN SINGLE MAMMALIAN CELLS

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ABSTRACT Lysis experiments demonstrate that unirradiated cell nuclei are rather stable in alkaline solutions (pH $\geq$12) in the sense that DNA from each cell keep together as a separate unit for more than 30 min. Irradiated cells lyse more quickly. Based on this, a preliminary method is devised involving the dye acridine orange and microfluorometry, whereby the relative amounts of single-stranded and double-stranded DNA can be estimated from each cell after alkali treatment. This extends the "DNA strand separation method" of estimating DNA strand breaks to the level of single cells.

INTRODUCTION

A sensitive method of measuring DNA breaks in individual cells should be of value when heterogeneous cell populations are studied or when only a small number of cells are available. We have observed that DNA strand separation in alkali may occur without dispersal of the DNA from each cell. This prompted us to try to develop a single-cell method. We report here our preliminary results.

RESULTS

Experiments designed to follow lysis of cells in alkaline solutions are reported in Figure 1 with legend. As shown in Figure 1a, the DNA is kept together in alkali as discrete units resembling swollen nuclei (the fine structure in the photomicrographs are artefacts from the filter). With increasing time in alkali, the "nuclei" are becoming more diffuse but the process is slow (not completed in 1 hr). The observation is not limited to the particular alkaline solution used in Figure 1; for example, in 0.1 M NaOH, 0.9 M NaCl, 0.01 M Na_2EDTA the situation is similar. Ionizing radiation given to the

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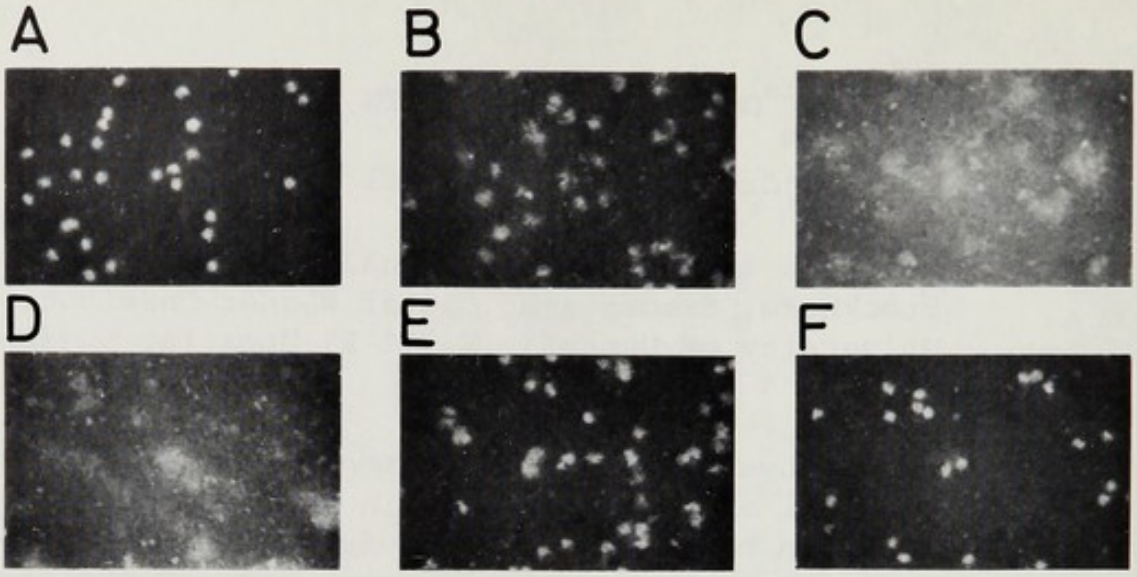

FIGURE 1. Damage-dependent lysis of Chinese hamster cells in alkali. A. Control B. 250 rad (^{60}Co irradiation) C. and D. 750 rad E. 750 rad +15 min repair (37°C) F. 750 rad + 30 min repair. About 10^5 cells were rapidly mixed with 20 ml alkali (0.03 M NaOH, 0.01 M Na_2HPO_4, 0.9 M NaCl) in the sample funnel of a membrane filter holder equipped with a 0.4 µm Unipore filter (47 mm diameter,matte side up). After 15 min in the dark, the "lysate" was filtered on to the filter (using vacuum). The filter was air dried, and pieces taken from central parts were mounted in 10 µg/ml of propidium iodide (a DNA intercalating fluorochrome) in phosphate buffered saline, PBS (pH 7.4). The distribution of fluorescence was observed and photographed in a Leitz Orthoplan microscope equipped with an incident light Ploemopak fluorescence illuminator (filter block M). One millimeter corresponds to 20 µm.

cells results in an accelerated "lysis" as seen in Figure 1b and 1c. Incubation of the cells at 37°C after irradiation reverses this effect, see Figure 1d-1f. It has not yet been determined to what degree double-stranded and single-stranded DNA (and perhaps RNA remaining after alkali treatment) contribute to the patterns seen in the figure.

A similar conclusion can also be reached by direct observation of cells in alkaline solutions containing ethidium bromide or propidium iodide as fluorescent marker for DNA, provided that shear forces are avoided and the observation is done before the exciting light of the fluorescence microscope has damaged the cells.

The effect demonstrated in Figure 1 may be used to estimate qualitatively radiation damage (probably DNA breaks, see below). In an effort to develop a quantitative method, we next wanted to estimate single-stranded and double-stranded DNA of

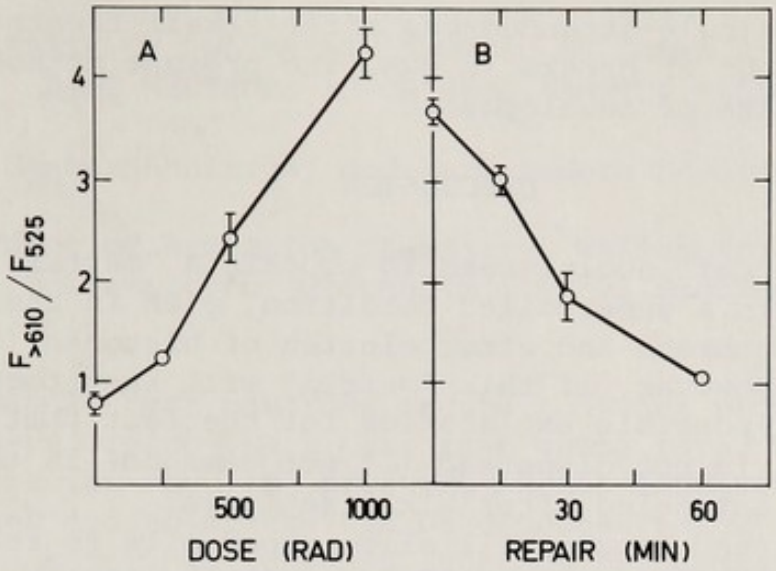

FIGURE 2. Estimation of DNA strand breaks in single cells. Intensity of red fluorescence ($F_{>610}$) divided by green fluorescence (F_{525}) from acridine orange (AO) in single cells (not corrected for different efficiences in the red and green) A. vs dose B. vs time of repair at 37°C after 1000 rad of ^{60}Co irradiation. Each point marks average and standard error of the mean for 10 individual cells. Cells at 0°C were mixed with agarose (0.67% in PBS, held at 42°C) and samples, containing 10^3-10^4 cells, were immediately spread out as thin layers on microscope slides (pretreated by being dipped in 0.25% agarose and air dried). After allowing the gel to form (at 0°C) the slides were immersed in alkali (composition as in Figure 1) for 30 min in the dark, neutralized in PBS for 10 min, washed in distilled water (10 min) and air dried. The preparations were mounted in 10 µg/ml of AO in 0.25 M sucrose, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4 (2) (giving excess of AO over DNA phosphate). Cells were located under phase contrast optics and their fluorescence measured in a Leitz MPV2 microscope photometer(Ploemopak, block H2) using a Leitz 610 nm barrier filter (red) and a 525 nm selection filter (green).

individual cells after alkali treatment. The fluorescent dye acridine orange (AO) has been used previously for this purpose after thermal denaturation in situ (1,2). It is very suitable as it emits green fluorescence when bound to double-stranded DNA and red fluorescence when bound to single-stranded DNA (and RNA). Since the membrane filter gave a high background with AO, we developed the agarose inbedment method devised in the legend to Figure 2. As is shown, the relative amount of red fluorescence in each cell was increased in proportion to dose of ionizing radiation given to the cells, and the effect was reversed by incubation at 37°C after irradiation. Like the

"DNA strand separation method" in bulk (3,4) we propose that the degree of single-strandedness after alkali treatment reflects the number of breaks in DNA. The present method is in a preliminary stage of development.

DISCUSSION

Mammalian cell nuclei seem to contain a "matrix" that withhold DNA, in a supercoiled condition, even in the absence of a nuclear membrane and after elution of histones (5,6). It is not known, however, if this "matrix" will keep together at pH 12. Another possible explanation for the fact that the DNA from each cell is not dispersed (if not damaged) is that the DNA is simply entangled after alkaline lysis.

Single-strand breaks will allow supercoils to relax (6) and will also promote DNA unwinding (3,4) in alkaline solutions. It is conceivable that this will cause the accelerated swelling and dispersal of DNA as seen in irradiated cells.

It seems possible that the "complex" described by Elkind and coworkers, which contain DNA and lipid (7), is related to the undissolved "nuclei" described here. For the "DNA strand separation method" in bulk (3,4), our findings imply that unwinding of DNA occurs to a large extent independently for each cell.

Single-strand breaks in DNA may either be directly induced by damaging agents or enzymatically induced by the cells during repair of various lesions. Since cell populations available for study are generally heterogeneous in several respects, a single-cell method should be useful in elucidating repair phenomena.

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REPAIR OF DNA BREAKS INDUCED BY GAMMA RAYS AND FAST NEUTRONS IN CHINESE HAMSTER CELLS.[1]

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ABSTRACT Fast neutrons induce a higher proportion of slow-rejoining DNA breaks than gamma rays. Long time of incubation results, however, in non-linear dose response curves and no breaks can be detected below a threshold, 5 krads for neutrons and 10 krads for gamma rays.

INTRODUCTION

In an earliar investigation we compared fast neutrons and gamma rays with regard to induction and repair of DNA breaks in hamster cells (1). Neutrons were less efficient, by a factor of 4, in producing breaks compared to gamma rays. More breaks remained, however, unrepaired after a few hours of incubation in the case of neutron irradiation. Recently Ritter et. al. presented an investigation where they studied DNA repair over a wide LET range (2). They found that the relative yield of non-rejoining DNA breaks were in close agreement with figures for single hit cell killing and suggested that non-rejoining DNA breaks constitute the predominant lethal event for high-LET radiations.

DETERMINATION OF DNA BREAKS IN MAMMALIAN CELLS

The method for measuring DNA breaks in cells is based on treatment of whole cells in alkaline solution for a limited period of time (1,3). Alkali causes double helical DNA to unwind and this process starts from free ends and strand breaks in the molecule. After neutralisation single stranded DNA is found to be present in direct proportion to the number of chain breaks in the DNA. Neutralisation is done in such a way that DNA is sheared to prevent a partial renaturation.

[1]This work was supported by the Swedish Natural Research Council.

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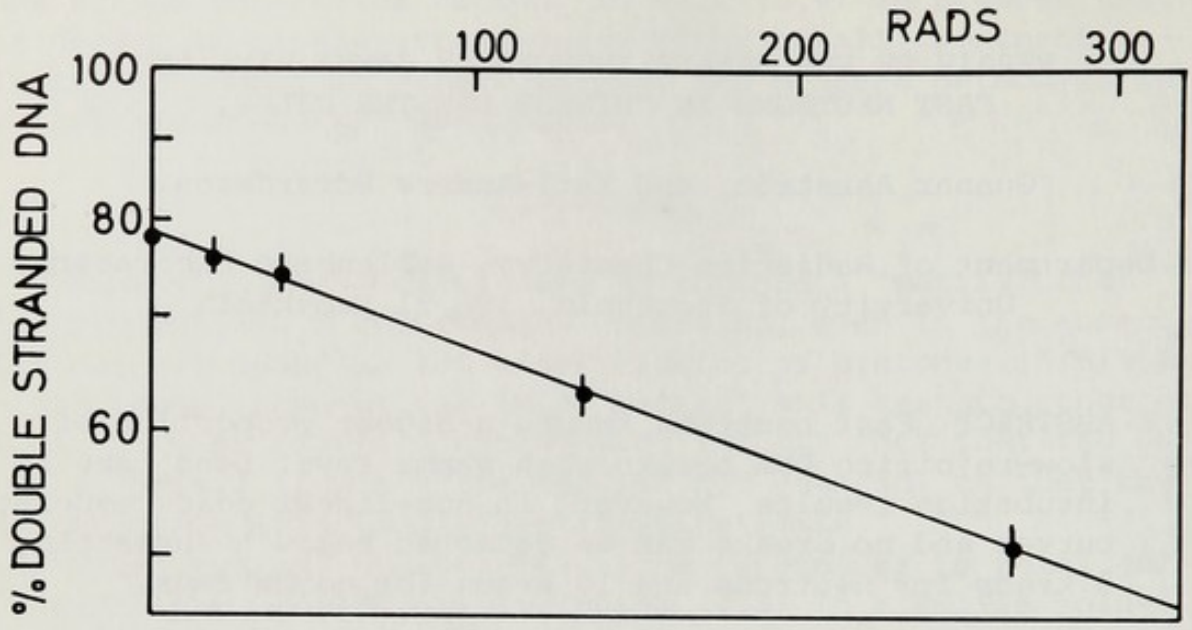

FIGURE 1. Survival of double stranded DNA in alkali.

^{3}H-thymidine labelled chinese hamster cells were irradiated with X-rays at 0°, washed free from medium and 10^5 cells lysed in 0.03 M NaOH, 1 M NaCl. The samples were kept in dark for 30 minutes at 20° and then neutralised by a forceful injection of 1 ml 0.02 M NaH_2PO_4 solution. They were then deep frozen after a short ultra sound treatment and addition of SDS (0.2 per cent final concentration). The samples were thawed again and loaded on small colums containing 200 mg hydroxyl apatite (Biogel HTP, selected batches). Single and double stranded DNA were eluted by 0.12 M and 0.25 M potassium phosphate buffer, respectively. Each point in the figure represents the mean of ten determinations done simultaneously. SEM is indicated. The number of chain breaks per rad was obtained by performing a sedimentation in an alkaline sucrose gradient using phage DNA as a molecular weight marker. For the lysing conditions given here we obtained the following expression (strand breaks in unirradiated cells included).

$$\text{Number of breaks per } 10^9 \text{ daltons} = -4.4 \log \frac{ds}{ss + ds}$$

ss = cpm eluted by 0.12 M buffer (single stranded DNA)
ds = cpm eluted by 0.25 M buffer (double stranded DNA)

Size of Mammalian Cell DNA. Alkali releases about 20 per cent of DNA from unirradiated cells in single stranded form (figure 1). This corresponds to 0.4 breaks per 10^9 daltons. One third of the breaks are due to alkaline degradation. We believe, however, that the remaining two thirds are breaks and alkali labile bonds present in the cell DNA. This means that we have single strand DNA molecular weights which by an order

of magnitude exceed those usually obtained by alkaline sucrose gradient methods. The rate of unwinding is high in the beginning but is continously slowed down and finally it is stopped. This happens probably when the single strand fragments have reached a size where interactions with other DNA molecules occur.The point of standstill is reached after about half an hour and further alkali treatment only increases the degradation as concluded from the fact that the slope of the dose response curve does not change. From figure 1 and the expression below we can calculate that one single strand break at maximum releases $4 \cdot 10^8$ daltons DNA in single stranded form, two fragments on each side of the break 10^8 daltons and a $2 \cdot 10^8$ dalton piece in the opposite strand.

DNA from mammalian cells will therefore not sediment as free single strand molecules in an alkaline gradient unless molecular weight is reduced by alkaline degradation or irradiation to such an extent that breaks in the same strand are at a distance of less than $2 \cdot 10^8$ daltons. The distribution of single stranded DNA released from incompletely degraded DNA will also appear to be nonrandom in size. This is because fragments above a critical size do not appear in free form.

REJOINING OF DNA BREAKS

DNA break repair in gamma and neutron irradiated hamster cells has been followed during incubation for 5 to 30 hours. Typical dose response curves are presented in figure 2. 15 and 24 hours repair are similar to 20 hours giving non-linear dose response curves. At 30 hours the picture is different, new breaks show up at lower dose levels and a nearly linear dose response is obtained. We feel that those breaks reflect some kind of DNA degradation related to interphase death.

Ionizing radiations induce at least two classes of DNA breaks, one class typical for low-LET radiations and which undergoes a fast repair in medium with a half-life of a few minutes (1). The other type of breaks induced in high yield by high-LET radiations are repaired at a much slower rate, several hours are required for a complete repair. We have also indications that there exist breaks which are repaired at an intermediate rate. An interpretation is that fast repair involves single strand breaks and the intermediate type of repair double strand breaks which have a structural support. Double strand breaks which occur between the nucleosomal bodies on the other hand require a three body collision to form a repair complex. Alternatively some structural rearrengements have to take place which will bring the loose ends together again.

There are good reasons to believe that slow-rejoining breaks are the most important lesions with regard to cell killing. As shown in figure 2, 3 breaks per 10^{10} daltons remain

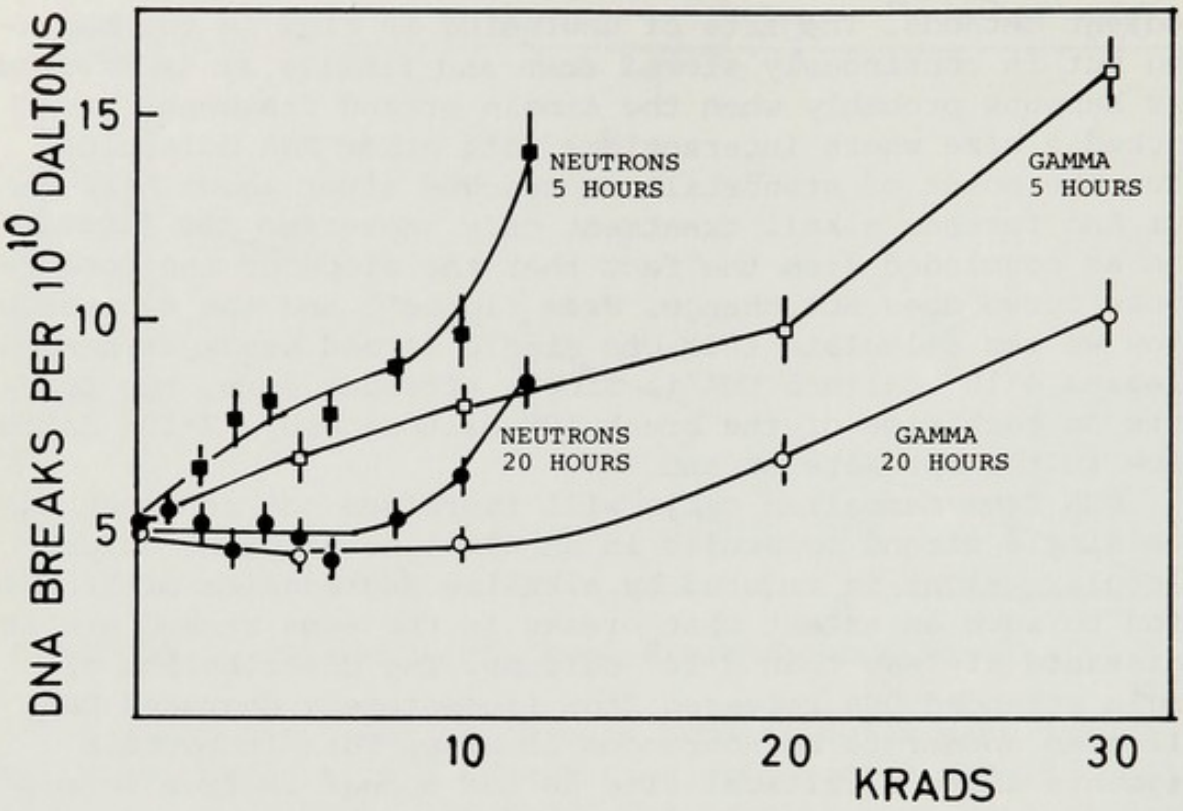

FIGURE 2. Rejoining of DNA breaks in hamster cells.

after 5 krads of neutrons and 5 hours of repair, i.e. around 20 breaks per cell per lethal hit. Slow-rejoining breaks cannot therefore be lethal hits per se. After 20 hours of repair no breaks are detected below 5 krads of neutrons. This means with the given resolution that less than 2 breaks per cell per lethal hit could be present. We can therefore not exclude the possibility that a fraction of breaks remain unrepaired and cause cell death. On the other hand we have not been able to detect differencies in strand break repair capacity between mouse lymphoma lines which do show a differential sensitivity towards fast neutrons. Our interpretation is that mammalian cells from a biochemical point of view are capable of rejoining in practice all types of DNA breaks. Mistakes in the reconstruction work occur, however, resulting in chromosomal aberrations lethal to the cell.

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A RELATIONSHIP BETWEEN DNA SINGLE-STRAND BREAKS AND DNA-PROTEIN CROSSLINKS IN INTERCALATOR-TREATED MOUSE L1210 CELLS

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ABSTRACT Alkaline elution methodology disclosed the appearance of DNA single-strand breaks and DNA-protein crosslinks in mouse leukemia cells treated with DNA intercalating agents. The breaks and crosslinks appear to be localized with respect to each other, and there is approximately a 1:1 relationship between the 2 lesions. The results exhibit a means of detecting proteins covalently linked to one terminus of single-strand breaks, as could under some circumstances result from the action of an endonuclease.

INTRODUCTION

A variety of chemical and physical agents induce DNA single-strand breaks and DNA-protein crosslinks in cells, and both of these types of lesions are often subject to repair or removal from the DNA (reviewed in ref. 1). We have recently noted in intercalator-treated L1210 cells an apparent association between these 2 DNA lesions (2). The results are suggestive of covalent linkage between DNA and enzymes capable of producing single-strand breaks.

METHODS

We used the technique of DNA alkaline elution (3) to quantitate both single-strand breaks and DNA-protein crosslinks. The quantitation of single-strand breaks was based on a calibration relative to the efficiency for single-strand breakage by X-ray. The measurement of DNA-protein crosslinks is based on the finding that proteins tend to adsorb to filters under alkaline elution conditions (4, 5). DNA single-strands that are held to filters through crosslinking to proteins can be dislodged by treatment with proteinase-K or by adding detergent to the eluting solution. The assay was characterized using trans-Pt(II) diamminedichloride, which initially produces DNA-protein crosslinks without detectable interstrand crosslinking or strand breakage (L. A. Zwelling, unpublished). The quantitation of DNA-protein crosslinks was based on the assumption that, under appropriate assay conditions,

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a DNA single-strand would be bound to the filter if and only if it is linked to one or more protein molecules. In these assays, drug-treated cells were exposed to 2-8 krad of X-ray in the cold in order to introduce a known frequency of random single-strand breaks. The alkaline elution patterns (fig. 1) exhibited 2 components, part of the DNA eluting very rapidly and part slowly. In cells exposed to only X-ray, between 3 and 7% of the cell DNA eluted in the slow component; this presumably reflects DNA-protein crosslinking since the slow component can be greatly reduced by proteinase or detergent. Cells treated with the trans-Pt(II) complex exhibited a concentration dependent increase in the slow component (fig. 1). The fraction of the DNA existing as single-strands crosslinked to protein was estimated by extrapolating the slow elution component to zero time. The dependences of the crosslinked fraction on drug concentration and X-ray dose can be formulated in terms of a simple probabilistic model, and the results agreed with theory. On this basis, it was possible to estimate DNA-protein crosslink frequencies.

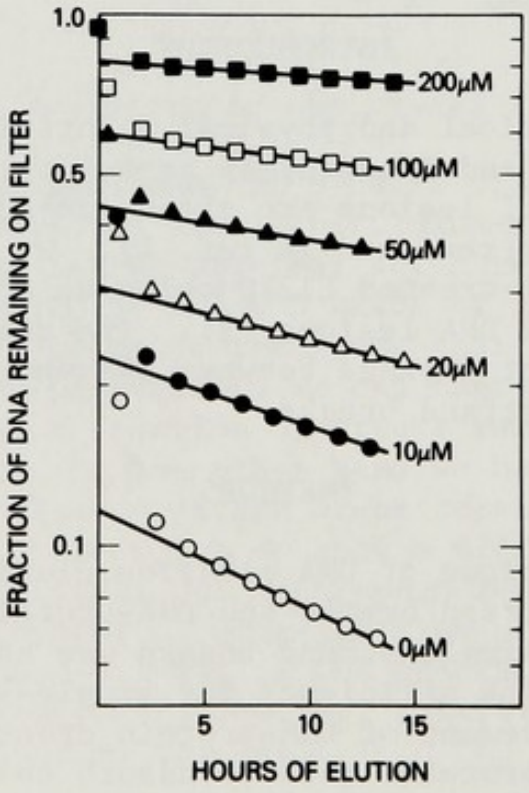

FIGURE 1. Illustration of DNA-protein crosslink assay in cells treated with trans-Pt(II) diamminedichloride. Analogous results were obtained with intercalator-treated cells. Log phase L1210 cells, pre-labeled for 1.5-2.0 doubling times with [2-^{14}C]-thymidine, were treated with various drug concentrations for 1 hr and then exposed to 3 krad of X-ray in the cold. The cell DNA was then analysed by alkaline elution.

RESULTS

These methods of analysis were applied to an unexpected circumstance which was found to exist in L1210 cells treated with DNA intercalating agents (2). The basic finding is illustrated in fig. 2 for the case of adriamycin. Although drug-treated cells did not, in direct assays (curve B), show any increase in elution rate to suggest the presence of single-strand breaks, marked increases in elution were disclosed in assay which included a proteinase-K step (curve G). This suggests that the drug induced the production of single-strand breaks which were hidden by protein. The increase in elution revealed in the proteinase assay was comparable to the effect produced by 300 R of X-ray (curve F). The elution in cells exposed to both adriamycin and 300 R (curve C) was reduced compared to 300 R alone (curve D), indicating the presence of crosslinks; these were inferred to be of the DNA-protein type since they were eliminated by proteinase (curve H, which approximated the additive effect of curves

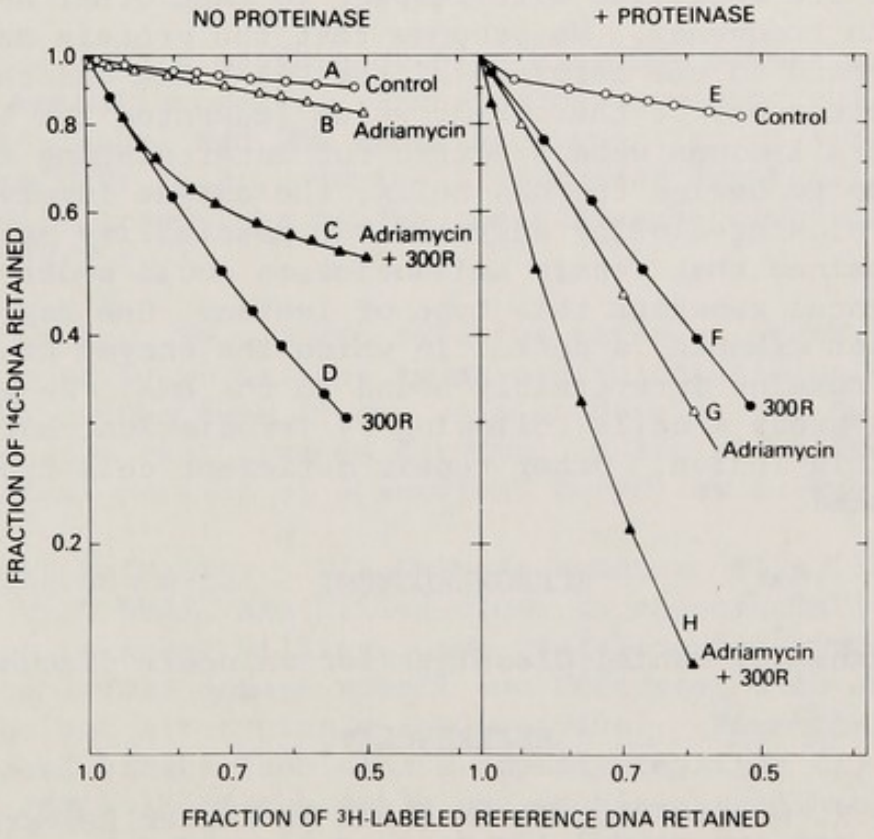

FIGURE 2. Evidence for protein-associated DNA single-strand breaks induced by treatment of L1210 cells with adriamycin. (2). Cells received either no treatment (curves A and E), or 2.8 μM adriamycin for 1 hr (curves B, C, G and H) and/or 300 R of X-ray (curves C, D, F, H). Assay were conducted either with (right panel) or without (left panel) proteinase digestion of the lysate (2).

F and G). The masking of the X-ray-induced breaks by adriamycin (curve C) however was much less striking than the essentially complete masking by the drug of its own breaks (curve B). We infer that the adriamycin-induced DNA-protein crosslinks are localized with respect to single-strand breaks also produced by the drug. Similar results were obtained with daunamycin, ellipticine, actinomycin, ethidium and miracil D, all of which are intercalating agents, but not with anthramycin or chromomycin, which are non-intercalative DNA binders. The frequencies of DNA-protein crosslinks produced by adriamycin and ellipticine were estimated by the method outlined in fig. 1 and related text. This frequency was found to be within a factor of 2 of the single-strand break frequency produced by the same treatment.

DISCUSSION

The results indicate that DNA intercalating agents induce single-strand breaks and DNA-protein crosslinks, and that the 2 lesions are localized with respect to each other and are similar in frequency. We propose that the protein may in fact be bound to one terminus of the DNA break and that the bound protein may be the enzyme which generated the break. Since these lesions were specific for intercalating agents, which tend to unwind the DNA helix, the enzyme involved may be a DNA nicking-closing enzyme. The possibility may also be entertained that repair endonucleases could under some circumstances generate this type of lesion. One may envision, for example, a defect in which the enzyme produces a nick but remains irreversibly bound to the DNA. We examined xeroderma group A cells following UV irradiation, but failed to find this lesion. Other repair deficient cell types remain to be tested.

ACKNOWLEDGMENT

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DNA DAMAGE AND MAMMALIAN CELL KILLING

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ABSTRACT DNA lesions--particularly single- and double-strand breaks--are induced in considerable excess by doses required to register one lethal "hit." In the case of X-ray cell killing, the disposition of such damage has been determined in the progeny of surviving cells. Fewer than 0.3% of the single- and 8.0% of the double-strand breaks are propagated. Hence, it is likely that in surviving cells all breaks are repaired. A mechanism of X-ray cell killing based upon misrepair of DNA breaks is proposed.

INTRODUCTION

Diverse agents capable of producing DNA damage are also lethal to mammalian cells. Focusing on surviving cells, I compare the lethal and DNA damaging properties of X-rays, ultraviolet (UV) light, and the fluorescent light (FL) exposure of cells grown in a medium containing 5-bromodeoxyuridine (BUdR), BUdR/FL.

DNA Damage. To compare relative rates of break induction (1), dose must be normalized to an equivalent biological effect. This can be done if the unit of dose is the D_o dose (i.e., the dose that reduces survival by 1/e along the exponential, final portion of a survival curve) as in Table I.

BUdR/FL Lethality. V79 Chinese hamster cells, labeled overnight with BUdR, are killed close to exponentially (9). In contrast to X-ray killing, dose fractionation gives no evidence of sublethal damage repair and treatment with anisotonic buffer does not alter single-dose survival. Nevertheless, single-strand breaks (ssb's) are rapidly repaired (2). It is estimated that $\sim$1% of the ssb's are double-strand breaks (dsb's) (4). But even if most of the latter are not frank scissions, it is probable that $dsb/D_o >> 1$.

X-Ray Lethality. X-irradiated cells rapidly repair sublethal damage. They may also suffer potentially lethal damage which they can repair even more rapidly (5). As with breaks induced by BUdR/FL, X-ray-induced breaks are rapidly repaired. However, X-ray-induced breaks either differ qualitatively, or

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are registered in different structures containing DNA since 1/50th as many are produced as by BUdR/FL.

TABLE I

DNA DAMAGE VERSUS SURVIVAL OF V79 CHINESE HAMSTER CELLS

Treatment	ssb[a]/D_o	ssb repair	dsb[a]/D_o	Survival curve	SLD[e] repair	PLD[f] repair (5)
BUdR/FL	50,000 (2)[b]	rapid	500 (4)	exponential	none	none
X-rays	1,000[c]	rapid	40[d]	threshold	rapid	rapid
UV	100 (3)	slow/none	<<ssb per D_o	threshold	slow	none

[a] ssb = single-strand break; dsb = double-strand break.
[b] Numbers in parentheses indicate references.
[c] Based on $D_o \simeq 100$ rads, 60 eV/ssb, and 1×10^{-11} g DNA/cell.
[d] Based on ∿25 ssb/dsb (Elkind, unpublished data; published values for mammalian cells range from 12 (6) to 46 (7), and recently ∿23 was reported for E. coli (8).
[e] SLD = sublethal damage; repair usually measured by dose fractionation.
[f] PLD = potentially lethal damage; enhanced damage expressed by postirradiation treatment with anisotonic buffer (5).

UV Lethality. The photolesions that appear as ssb's are repaired slowly after doses corresponding to high survival, >10%; after low-survival doses, the number actually increases (3,10).

A Search for Breaks in X-Ray Survivors. Although X-ray lethality very likely involves DNA damage (for review, see 11), it is clear from Table I that X-rays do not kill cells efficiently. To follow the fate of ssb's in mammalian cells by sedimentation, biologically large doses of radiation are invariably used. The superstructure of the DNA must be sufficiently damaged (e.g., 12) to facilitate the release of DNA whose sedimentation properties are readily interpretable. Consequently, studies of the repair of breaks suffer from several shortcomings: 1) the fate of breaks in dying, as opposed to surviving, cells is observed; 2) since the superstructure may reform during the process of repair, the completeness of repair may not be assessable; and 3) depending on the dose, quality of radiation, etc., as part of the pathology of a dying cell, DNA breakdown may set in so that repair becomes offset by DNA degradation. Hence, measurements of the rate or completeness of DNA repair may be confounded by artifacts.

To circumvent these problems, the following experiment

was performed. V79 cells were exposed to repeated ∿1000 rad X-ray doses. Between each fraction, cells were incubated to permit the survivors to multiply and the killed cells to lyse. After a total of 20 krads and again after 50.8 krads, surviving cells and their offspring were examined and compared to controls to determine: 1) if ssb's were inherited; 2) if the break-induction curve had changed; 3) if the rate of repair after a large dose was different; and 4) if the survival curve was altered. Only the results after 50.8 krads are described since they do not differ from those after 20 krads.

For control cells, 2.4 krads are required to produce one ssb/2×10^8 daltons (number average). Using the technique of the cosedimentation of DNA from control and test cells (13), it was found that: 1) the 21 ssb's in 2×10^8 dalton DNA due to 50.8 krads were not inherited; 2) the induction curve of new breaks was not changed; 3) 50.8 krad survivors repair ssb's equally as rapidly as control cells; and 4) although the 50.8 krad survivors were a little more resistant than controls, the D_o increase was modest, 36%. Of the 21 ssb's, 1.7 would have come from 0.85 dsb's; thus, repair of duplex scissions is also indicated. An analysis of the resolution available in the method shows that a difference of at least 0.07 ssb/2×10^8 daltons can be detected. Hence, fewer than 1:300 ssb's and fewer than 1:12 dsb's remained unrepaired following an accumulated dose of 50.8 krads.

Cell Killing Hypotheses. The foregoing does not indicate when breaks are repaired in surviving cells or their progeny. Nevertheless, it shows that radiation survivors repair the vast majority (if not all) of the breaks registered. Since the repair of DNA in lethally irradiated cells is rapid, it is probably rapid in surviving cells as well.

Whatever may be the molecular basis for a threshold on an X-ray survival curve, beyond the threshold a D_o dose registers one lethal "hit". In the ensuing competition between damage repair and damage fixation, this hit could be the one ssb or the one dsb that loses the race. Thus, even if we discount the effectiveness of ssb's produced by one D_o dose, because we assume that all such breaks are repaired faithfully by a pre-replicational process, we must conclude that, at least, one dsb fails to be repaired soon enough if at all.

A second hypothesis derives from the apparently large amount of repair that surviving cells effect. Whereas the lack of repair of one or a few ssb's and/or dsb's is not excluded, the limit of the unrepaired damage is sufficiently low to support the likelihood that all DNA breaks are repaired in surviving cells. As noted, lethally affected cells rapidly repair breaks. Consequently, the rejoining of breaks occurs in the entire population. This suggests the possibility that

lethality results from a misrepair of one or more ssb's or one or more dsb's per D_o. Since misrepair would apply to only 0.1% of the ssb's or to only 2.5% of the dsb's, it is clear that the repair of strand breaks is on the whole a faithful process.

CONCLUSION

The data discussed lead to two main conclusions. First, the induction of X-ray resistant cells is not a likely event or if it is, such cells are selected against. And second, most, if not all, of the double-strand breaks (as well as the single-strand breaks) are repaired in surviving cells. The latter conclusion is contrary to what has been generally believed. The ability of cells to effect double-strand rejoining makes understandable processes which involve major transpositions of genetic material (i.e., sister-chromatid exhanges) or less drastic rearrangements which may result in mutation or neoplastic transformation and represent, therefore, misrepair compatible with survival.

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REJOINING OF DNA STRAND BREAKS IN NONDIVIDING CELLS IRRADIATED IN SITU[1]

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ABSTRACT If the radiation dose of ^{60}Co γ-rays (LET_∞ = 0.3 keV/μm) or neon ions ($LET_\infty \sim 35$ keV/μm) is kept below that at which the electroretinogram is destroyed, retinal photoreceptor cells rejoin at least 99.9% of the strand breaks introduced into their DNA in situ; however, the rejoined DNA does seem to degenerate subsequently. Provided the radiation doses are kept within the prescribed limits, the ability of photoreceptor cells to rejoin DNA strand breaks is not influenced by the age of the animal.

INTRODUCTION

Although mammalian cells are capable of rejoining strand breaks introduced into their DNA by ionizing radiation, it is not known whether the rejoining is complete or accurate, and over the years a favored hypothesis has been that unrejoined strand breaks are lethal. In order to address these questions, it is necessary to study the responses of cells to doses of radiation from which they will survive. A similar rationale was used when it was established that Micrococcus radiodurans can rejoin all the breaks introduced into its DNA by anaerobic x-irradiation. Clearly, a cell that is dying is likely to suffer DNA degradation that could simulate unrejoined strand breaks, and, indeed, such seems to be the case with the very radiosensitive S/S variant of the L5178Y murine leukemic lymphoblast. Moreover, proliferating cells may amplify DNA damage simply by replicating their DNA, repairing their DNA, and so forth, and that amplification, which could

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be a consequence of cycle progression, might be misinterpreted as the incomplete rejoining of strand breaks. So, in order to examine the true potential(s) of the mechanism(s) that rejoin(s) strand breaks in the DNA of mammalian cells, it is necessary to follow the responses of post-mitotic cells to relatively small doses of radiation. For us, the cells of choice have been the neurons of the central nervous system (CNS), irradiated and maintained in situ. Reorienting-gradient zonal ultracentrifugation in alkaline sucrose gradients was used for analysis since the use of conventional rotors was precluded by the gelation artefacts and other anomalies that occur in those rotors.

RESULTS AND DISCUSSION

When the eyes of rabbits are irradiated with ^{60}Co γ-rays (LET_∞=0.3 keV/μm) the massed electrical response of the retina, which is expressed as the b-wave of the electroretinogram, is lost precipitately at a (critical) dose in the range 4,000-5,000 rads. The same is true when the ionization density of the incident radiation is raised by using neon ions (LET_∞∿35 keV/μm) or argon ions (LET_∞∿90 keV/μm). Complementary investigations on the fate of the DNA strand breaks in the retinal photoreceptor cells have also been completed with the former two radiations and the findings are similar: after doses of radiation below the critical dose more than 99.5% of the DNA strand breaks are rejoined, although the process takes several days, and the cells survive; after doses of radiation above the critical dose the DNA strand breaks are not rejoined and the cells degenerate. Following doses of 3,000-4,000 rads, the sensitivity of the analytical technique is such that the figure for the efficacy of the rejoining of DNA strand breaks can be increased to greater than 99.9%. In other words, within the limits of experimental sensitivity, all the strand breaks in the DNA are rejoined, and this despite the fact that the ratio of double strand breaks to single strand breaks should increase when the LET_∞ is raised from 0.3 keV/μm to 35 keV/μm.

Since the CNS usually receives its full complement of fully differentiated neurons at the birth of the animal, or shortly thereafter, and since damaged neurons are not replaced, the above observations have permitted us to address further questions: does the rejoined DNA remain intact throughout the subsequent life-span of the animal; is the broken DNA rejoined as effectively when irradiation is performed at different times throughout the life-span? These questions, of course, are related to another: does DNA damage accumulate in the neurons of the aging CNS? Our studies in

these areas are not complete, mainly because of the length of the median life-span of the rabbit (circa 7 years), and only studies with ^{60}Co γ-rays will be described here.

The size of the DNA in retinal photoreceptor cells does seem to undergo small decreases in size during the life-span of the rabbit (Figure 1); however, when rabbits were irradiated with 3700 rads of ^{60}Co γ-rays at different parts of the life-span (ages: 6 weeks; 6 months; 3.5±0.5 years; 4.5±0.5 years; 7.0±1.0 years) no differences in the rate of rejoining, or the extent of rejoining, of DNA strand breaks were observed (1) - in agreement with the results from earlier experiments on cerebellar neurons (2). Long-term studies have to be restricted to lower doses because of the degeneration of tissues proximate to the retina. Results obtained when 6-week-old rabbits were irradiated with 1,000 rads of ^{60}Co γ-rays, and

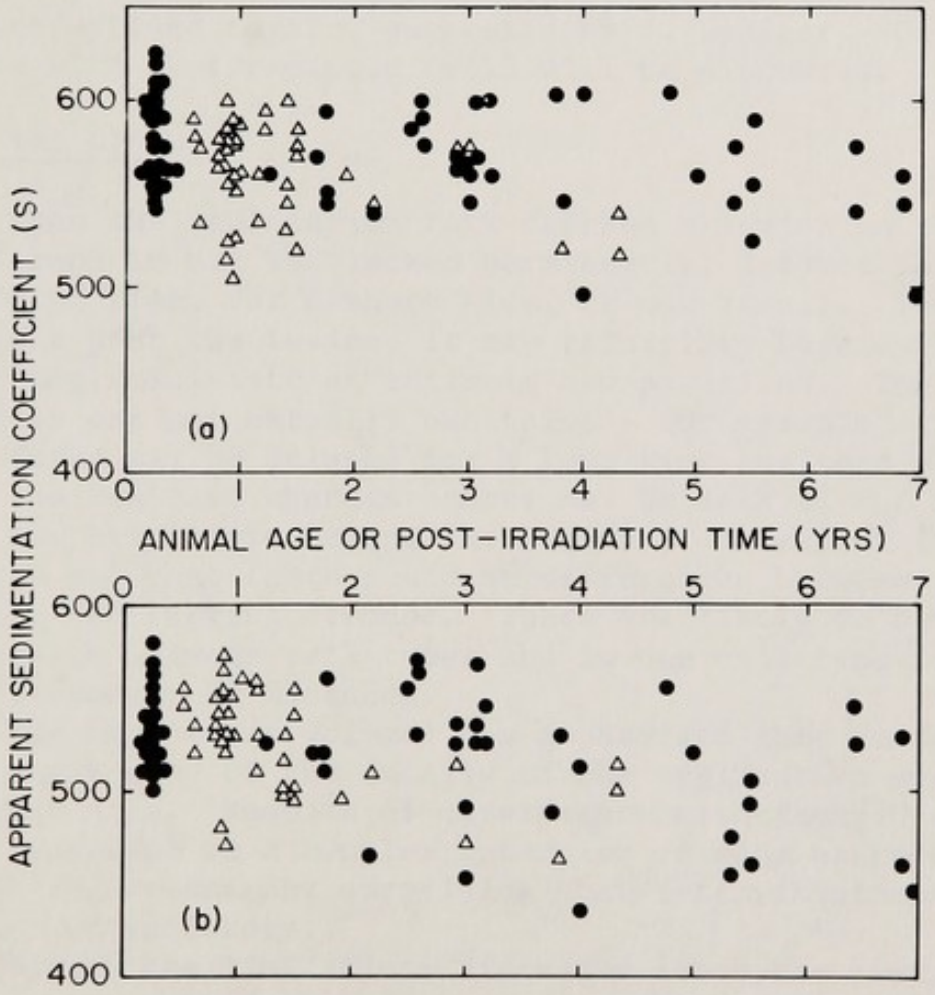

FIGURE 1. Sedimentation of the DNA from the photoreceptor cells of the rabbit retina. Aging animals, ●; animals irradiated with 1,000 rads of ^{60}Co γ-rays when they were 6 weeks old, Δ. (a) Peak sedimentation coefficient; (b) Mean sedimentation coefficient.

the retinas analyzed as the animals aged, are shown in Figure 1. Although this experiment has been in progress for only 4 years, the current results do suggest that the rejoined DNA degenerates subsequently.

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WORKSHOP SUMMARY: Replicative Bypass Mechanisms in Mammalian Cells.

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The way in which mammalian cells are able to replicate DNA using damaged templates continues to be a controversial subject. Eventual synthesis of high molecular weight DNA in damaged cells has been termed postreplication repair, replicative repair or bypass repair. All of these terms imply a particular kind of mechanism, which may not necessarily be generally applicable. I shall therefore use the term daughter-strand repair, suggested by J. Doniger. Only experiments with UV-irradiated cells will be discussed.

Gaps and blocks

When the replication fork reaches a lesion on the parental strand it may be blocked permanently, delayed for a long period of time, for a short time, or not at all. The fork may continue past the lesion, it may reinitiate beyond the lesion or it may reinitiate at entirely new positions. These possibilities are not mutually exclusive - for example, in one cell some forks may be delayed for a long time and then proceed past the lesions, whereas others may be delayed for a short time and reinitiate beyond the lesions. Meneghini (1) has pointed out that there could be differences between the leading and lagging strands. There are likely to be differences both between cell types and in one cell type irradiated with different UV fluences.

Edenberg (this volume) has emphasized that we are ignorant about many of the details of DNA replication even in undamaged cells. Results of experiments with damaged cells are thus dependent on a complex interplay of many unknown factors, and it is perhaps not surprising that interpretations often appear contradictory.

Within the limitations discussed above, the results from our laboratory using xeroderma pigmentosum (XP) variants (2), and of J. Doniger, using V-79 hamster cells (this volume), suggest that under the conditions used in these experiments gaps can be detected in daughter DNA strands after UV-irradiation. On the other hand Edenberg (3) has studied DNA replication in irradiated HeLa cells by fibre autoradiography, and he has suggested that replication forks are blocked for a

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long time at lesions and do not reinitiate beyond them. In this volume he has cited results from other systems, which are consistent with this hypothesis. Several other observations with different systems cannot however be easily fitted into his model. In some cell types, for example, the DNA strands synthesized soon after irradiation are considerably longer than would be predicted if pyrimidine dimers blocked the progress of the replication forks (e.g. see ref. 4 and results with normal human cells in ref. 2). It is evident therefore that one cannot necessarily extrapolate results from one set of experimental conditions to cover all situations, and one should be wary about drawing dogmatic conclusions.

Exchanges of Pyrimidine Dimers

Daughter strand repair in Escherichia coli is associated with gross recombinational exchanges (5) and equilibration of the pyrimidine dimers between parental and daughter strands (6). Gross sister-strand exchanges have not been observed in mammalian cells, but several workers have detected the association of endonuclease-sensitive sites (pyrimidine dimers) with DNA synthesized immediately after irradiation (e.g. see 7). The frequency of these dimer sites associated with the daughter strands (DSDS) is about 10-20% of that in the parental strands. Meneghini has raised the possibility that DSDS could result from the elongation of daughter strands present before irradiation (and therefore containing dimers) with labelled DNA synthesized after irradiation. This has been tested in three different laboratories by three methods: (A) increasing the time interval between irradiation and labelling,(B) irradiating synchronized cells in G1 so that no daughter strands existed at the time of irradiation, and (C) separating the DNA synthesized after irradiation by density labelling. Once again contradictory results have been obtained. Using method (A) we concluded that many if not all DSDS were the result of elongation (8), as did D'Ambrosio and Setlow using methods (A) and (B) (this volume). On the other hand Meneghini et al (this volume), using all three methods, obtained evidence for bona fide transfer of pyrimidine dimers into the daughter strands. This issue therefore also remains to be resolved. There does however seem to be agreement that if exchange of dimers is a real phenomenon it is confined only to the DNA synthesized during the first few hours after irradiation, (see also van Zeeland and Smith, this volume).

Recovery of the ability to synthesize normal sized DNA after UV-irradiation

In those cells in which the DNA synthesized at early times after UV-irradiation is smaller than that in unirradiated cells, the DNA synthesized at later times after irradiation is of normal size (9). This has been observed in rodent, normal human, XP and XP variant cells. Meyn has studied this phenomenon in more detail in Chinese hamster CHO cells (this volume) and has found that this recovery process is not inhibited by cycloheximide, and does not therefore appear to be an inducible process. It is not observed if the cells are irradiated in G1.

Recovery of the ability to synthesize DNA at normal rates

It is important to bear in mind that the ability to synthesize normal-sized DNA is not necessarily accompanied by an ability to synthesize DNA at normal overall rates. Rudé and Friedberg (10) recently reported that normal human cells recovered the ability to synthesize DNA at normal rates six hours after irradiation. This recovery was not however seen in an excision-defective XP cell. Cells from a sun-sensitive child (11961), and from patients with Cockayne's syndrome are sensitive to UV but have normal excision (11, 12) and daughter strand repair in standard experiments. We have found (this volume) that,like XP cells,they do not recover normal rates of DNA synthesis several hours after irradiation. It thus appears that this recovery process is not correlated with any of the other repair or recovery processes observed in human cells. Its mechanism is as yet obscure but it seems to be of crucial importance for the survival of the cell.

XP variants

Daughter strand repair is defective in XP variants. It takes much longer than in normal cells and is inhibited by caffeine (2). Fujiwara (this volume) has been able to subdivide twelve XP variants into three categories which differ by the degree of potentiation of UV-induced cell killing by caffeine; this is correlated with the severity of the defect in daughter strand repair, and the inhibition of daughter strand repair by caffeine.

Conclusions

Studies on replication of DNA in damaged cells present us at the moment with a rather confused picture and many unresolved questions. It is hoped that future work will clarify these issues and elucidate the relationships between the various repair and recovery processes which enable mammalian cells to replicate damaged DNA.

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DNA REPLICATION IN ULTRAVIOLET-IRRADIATED MAMMALIAN CELLS

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ABSTRACT Data presently available support the hypothesis that inhibition of DNA replication in ultraviolet-irradiated mammalian cells is due to long pauses when replication forks encounter pyrimidine dimers in the template DNA.

INTRODUCTION

While it has long been known that ultraviolet light (UV) inhibits DNA replication in mammalian cells, there is still controversy over the mechanism of this inhibition. This is not surprising, since detailed models of replication on UV damaged templates are difficult to evaluate in light of our still insufficient knowledge of DNA replication in undamaged cells. Nevertheless, a consideration of the most likely hypotheses is important to both the design and evaluation of experiments in which DNA replication after UV is examined.

The original model of replication on UV-damaged templates was that the replication process skipped past dimers in the parental DNA and continued beyond these lesions, after leaving gaps of about 1000 nucleotides (1). A second model proposed that replication forks halt when they reach pyrimidine dimers in the DNA (2,3), and that synthesis beyond the dimer is delayed for relatively long times.

DATA AND DISCUSSION

Data which have accumulated since these models were proposed are generally consistent with the second hypothesis, that the inhibition of DNA synthesis by UV is due to relatively long pauses (60-90 min) when replication forks reach dimers (4,5). The kinetics of DNA replication after UV irradiation of HeLa cells (3,4) and human fibroblasts (6) are consistent with this model. The accumulation of replication forks after UV (2,7) is also consistent with this model. In mammalian mitochondria (which lack repair capability) replication continues only until a dimer is reached and then halts (8); the arrested replication intermediates accumulate and

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remain for at least 48 h. Recent studies on SV40 replication in the nuclei of monkey cells (9,10) also indicate that dimers act as blocks to replication forks: Sarasin & Hanawalt (9) have shown that after UV irradiation, replicative intermediates accumulate with daughter strands of the size and in the location predicted if bidirectional replication began at the normal origin and proceeded only to the first dimer in each direction. They estimate a pause of 40-60 min before synthesis resumes and continues up to the next dimer (9).

DNA fiber autoradiography was used to show that in HeLa cells, DNA synthesis in each replicon continued at its previous rate until a dimer is reached (4). ^{3}HdT was added to cells immediately after irradiation and samples were taken for DNA fiber autoradiography at various times. Unirradiated cells showed a linear increase in the mean length of DNA segment synthesized. Cells irradiated with 10 or 17.5 J/m^{2} showed an increase in mean segment length for only 30 min, and no further increase during the next 60 min. [It should be noted that a unimodal distribution of segment lengths was observed, ruling out the idea that a fraction of replicons halted while others continued unaffected.] This is the pattern predicted if dimers block replication forks; it is not consistent with replication forks skipping past dimers and continuing synthesis, nor with a general slowing of the rate of fork progression. A different study confirmed that no change in fork movement rate was involved (11). The inhibition due to blocking replication forks was sufficient to account for the decrease DNA synthesis (i.e., effects on replicon initiation, if any, were responsible for only a minor part of the inhibition of DNA synthesis (4)).

A new study of Chinese hamster cells by fiber autoradiography (12) purports to reach the opposite conclusion, namely that DNA synthesis proceeds rapidly past dimers. However, the criteria used for selecting the fibers to be measured (tandem sets of symmetrical pairs) would virtually preclude measuring replicons in which a fork is blocked by a dimer. Doniger (12) does conclude that the inhibition of DNA synthesis by UV is due to non-functioning replicons; these could well be replicons at which synthesis is halted at dimers (i.e., replicons not measured under his criteria).

The observation that DNA synthesized at short times after UV is in sizes approximately equal to the interdimer distance on the parental strands is predicted by both models (4,13).

It has not yet been possible to determine whether a dimer blocks synthesis of only one daughter strand or of both; data from DNA-fiber autoradiography, for example,

are intermediate (4). This might be a reflection of the asymmetry of the replication fork. That is, a dimer opposite the leading (continuous, 5'-3') strand might block synthesis on both strands, while one opposite the retrograde (discontinuous, 3'-5') strand might only block completion of a single Okazaki fragment (a possibility discussed by Meneghini (14)). This hypothesis shares most of the predictions for dimers blocking replication, though when a dimer is first encountered opposite the retrograde strand it predicts that a small gap, averaging half the size of an Okazaki fragment (i.e., about 100 nucleotides (15)) would be left before the replication fork halts at the first dimer opposite the continuous strand. Such gaps should be much smaller than the 1000 or more nucleotides previously reported (1,13,16). It also predicts that the length of DNA synthesized after UV would be intermediate between that predicted if a dimer stopped synthesis only of one daughter strand, or of both.

The hypothesis that dimers block replication forks for long periods points out the need for caution in interpreting experiments on "postreplication repair" in mammalian cells. One problem is that labeling DNA after irradiation predominantly results in end-labeled molecules (since replication pauses at dimers), making interpretation of alkaline sucrose gradients potentially misleading. Another problem is that pulse label given even some time after irradiation can be added to the ends of replicons which had begun synthesis before UV, but which were held up by one or more dimers. This would lead to the finding that the size of DNA made after irradiation was sensitive to a UV-specific endonuclease, a finding that has been interpreted as showing recombination of dimers into daughter DNA (17).

Two additional points are worth noting here, although they are quite speculative. First, although it seems likely that the dimer itself blocks replication (since a pyrimidine dimer would probably not code properly for an incoming nucleotide and thus might stall an accurate replication complex), the possibility also exists that it is the interaction of the lesion with some component of the cell's repair "machinery" which creates the block to replication. This would not change the predictions of the models discussed, but might be relevant to differences in post-UV DNA replication in different cell lines.

A second problem, as yet unsolved, is how the replication machinery can eventually continue synthesis beyond dimers, and can apparently "ignore" dimers which remain in the DNA at long times after UV (i.e., after 6-8 h, dimers do not apparently block DNA synthesis (14)). A highly specula-

tive model might be that some cellular component binds to or otherwise alters the dimer (or alters the replication machinery) such that the dimer can code for its complementary nucleotides, without changing the chemical identity of the dimer when extracted DNA is analyzed. This idea could be tested when the enzymology of DNA replication is far better understood, and well defined in vitro DNA replication systems have been fully characterized.

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PYRIMIDINE DIMERS IN DNA STRANDS OF MAMMALIAN CELLS SYNTHESIZED AFTER UV-IRRADIATION[1]

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ABSTRACT Two different experimental procedures have been utilized in order to ascertain whether pyrimidine dimers found in DNA daughter strands are due to recombination or were introduced during UV-irradiation. The results show that 10-20% of the dimers are indeed transferred from parental to daughter strands.

INTRODUCTION

Recently, we have shown that DNA synthesized after UV-irradiation in HeLa and normal human diploid cells contains sites sensitive to UV endonuclease (1,2). The simplest interpretation for this fact was that dimers were transferred from the parental do the daughter strand by a recombinational mechanism, and that this process played a role in the dimer circumvention. These results were confirmed in normal human cells and extended to xeroderma pigmentosum (XP) (3,4 and A. Lehmann, personal communication) and chinese hamster cells (5). However, alternative interpretations had not been ruled out: since in eucaryotes there are always replicons varying in size from zero to that of a full replicon (6), the dimers could have been introduced in the daughter strand during irradiation. Later on, this DNA would be elongated during the radioactive pulse, and because of the pre-attached dimers, the labeled DNA would be sensitive to the UV endonuclease. A second possibility is that repair replication occurs during the radioactive pulse, thus introducing labeled DNA in the parental strands. Both these possibilities had to be carefully considered before concluding that DNA exchange does, in fact, occur.

RESULTS

Several experiments have indicated that repair replication can lead to an overestimation of dimer transfer from parental to daughter strands in excision-proficient cells. We therefore decided to work with XP cells since separate experiments, in which the CsCl density gradient technique was employed, re-

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vealed that the level of repair replication in these cells after a 12.5 J/m^2 fluence averaged at most 2% of the semi-conservative replication. With such a low level of repair replication, its influence on the estimation of dimer transfer is negligible. The experiment of Fig. 1 shows the action of

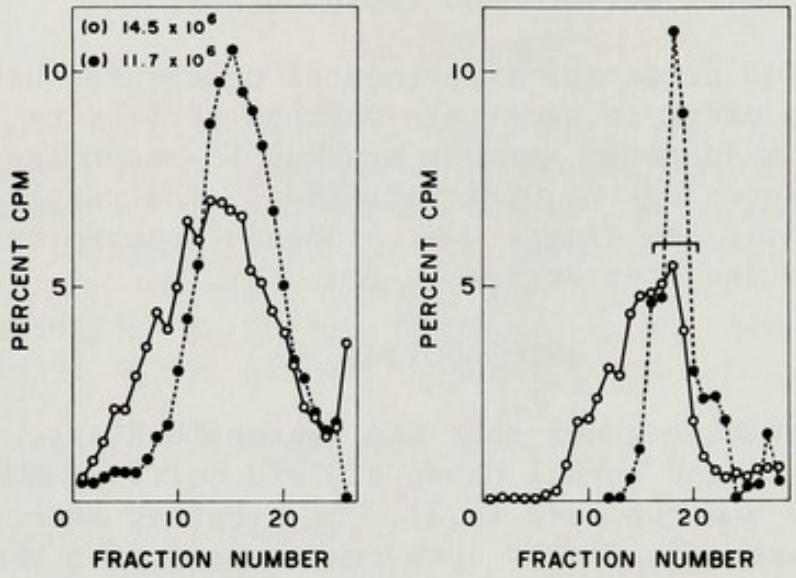

Figure 1. Action of *M. luteus* UV endonuclease on DNA synthesized after UV-irradiation. SV-transformed XP12RO (complementation group A) cells were irradiated with 12.5 J/m^2 of 254 nm light, pulse labeled for 4 hours with 10 μCi/ml ^{3}H-thymidine and chased in radioactive-free medium for 20 hours. The DNA was extracted, incubated with (●) or without (0) an extract of *M. luteus* corresponding to fraction II of Carrier and Setlow (7) and centrifuged in alkaline sucrose gradients. DNA extraction, centrifugation and calculation of number average molecular weight (figures on the upper left corner) were carried out as previously described (8).

Figure 2. Separation of daughter DNA entirely synthesized after UV irradiation. XP cells were incubated with bromodeoxyuridine (Budr), irradiated and labeled as described in the text. The DNA was extracted and centrifuged in CsCl density gradient (6.5 ml, initial density 1.715 g/cm^3) in 50 Ti rotor (Spinco) at 20°C for 36 hours at 37000 rpm. ^{14}C-DNA was added as a marker. The indicated fractions were pooled for experiments described in Table I; (0) ^{3}H-DNA; (●) ^{14}C-DNA.

M. luteus endonuclease on DNA from XP cells labeled after irradiation. A drop in molecular weight is observed, indicating the presence of pyrimidine dimers in the daughter strands. The results of Table I give an estimate of the specificity of

the *M. luteus* extract. The background of non specific action (row A) is relatively low and has been discounted in all cases in order to obtain the actual number of sensitive sites. The daughter strand has 15% as many sensitive sites as the parental strand, which may be attributed to pyrimidine dimers if we assume the specificity of the extract for this lesion.

As discussed above, the presence of dimers in the daughter strand might be due to recombination (transferred dimers) or to irradiation of replicons which had initiated replication prior to the irradiation (pre-attached dimer). In order to evaluate this latter possibility, two procedures have been devised:

1. Synchronized cells were blocked at the entrance of the S phase with hydroxyurea. No previously initiated replicons are present and thus there should be no pre-attached dimers in the daughter strands.

TABLE I

NUMBER OF SITES SENSITIVE TO *M. LUTEUS* ENDONUCLEASE

Type of experiment	Origin of DNA	Number of sites/10^7 mean and s.e	Number of experiments
A-	Unirradiated cells	0.02 ± 0.02	10
B-	Parental DNA from irradiated cells	1.00 ± 0.18	4
C-	Daughter DNA from irradiated cells	0.15 ± 0.07	5
D-	Daughter DNA from synchronized cells	0.12 ± 0.03	4
E-	Daughter DNA entirely synthesized after irradiation	0.20 ± 0.08	4

The number of sites sensitive to UV endonuclease was determined as previously described (8). In B and C, the cells were labeled before (B) or after (C) irradiation with 12.5 J/m^2. In D, the cells were incubated for 18 hours in medium containing 5 mM hydroxyurea, resupplied with fresh medium for 10 hours, and blocked at the G_1-S transition by incubating for 24 hours with 5 mM hydroxyurea. The cells were then irradiated with 20 J/m^2, resupplied with fresh medium, labeled for 2 hours with 10 µCi/ml ^{3}H-thymidine and chased in radioactive-free medium for 18 hours. In E, fractions corresponding to the peak of Fig. 2 were pooled, dialyzed and assayed as described in legend of Fig. 1.

The final figure of $0.12/10^7$ dimer sites in the daughter strands found with this protocol (Table I,D) should therefore represent the actual number of dimers transferred from parental to daughter strands.

2. Another procedure was to incubate the cells in medium containing 10^{-5}M bromodeoxyuridine and 10^{-6}M fluorodeoxyuridine for 2 hours before irradiation with 12.5 J/m^2. The cells were then pulse-labeled for 4 hours with ^{3}H-thymidine and chased for 20 hours in radioactive-free medium. The extracted DNA was centrifuged in CsCl density gradient and fractions from the normal density peak (Fig. 2) were pooled, dialyzed, and assayed for the presence of dimers. All the daughter DNA synthesized before irradiation will contain bromodeoxyuridine and be in a denser position. The number of dimers present in the normal-density daughter DNA, which represents DNA synthesized exclusively after irradiation, was $0.20/10^7$ (Table I,E).

DISCUSSION

The two sets of experiments described here have shown that DNA daughter strands synthesized after UV-irradiation contain dimers which could only have been transferred from parental DNA by a recombinational process. The observed number of dimers transferred varied somewhat between the two methods. In particular, the number of dimer sites in daughter strands determined by the bromodeoxyuridine pre-labeling procedure was higher than that determined directly (Table I,C), which should correspond to the sum of both transferred and pre-attached dimers. The presence of Budr-containing DNA during irradiation may have stimulated recombination or the procedure itself may have given more weight to those molecules which elongated more successfully during the radioactive pulse. In any event, we may conclude that about 10-20% of the dimers are transferred from parental to daughter strands. It is possible, although not certain, that the transfer of dimers is involved in the replication bypass of these lesions, as is the case in the bacterial system (9,10). If so, this low rate of transfer as compared to bacteria indicates that another non-recombinational process is involved in the dimer bypass in mammalian cells.

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ON THE PRESENCE OF UV-ENDONUCLEASE SENSITIVE SITES IN DAUGHTER DNA OF UV-IRRADIATED MAMMALIAN CELLS

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ABSTRACT Asynchronous Chinese hamster cells (V79) were irradiated with 10 Jm^{-2} UV radiation and 0.25 to 4 hours later pulse-labeled with [^{3}H]thymidine. Cells synchronized by shaking off mitotic and G_1 cells were irradiated in either the G_1-phase or S-phase of the cell cycle and pulse-labeled with [^{3}H]thymidine in the S-phase. After a 12-14 hour chase in unlabeled medium, the DNA was extracted, incubated with Micrococcus luteus UV-endonuclease and sedimented in alkaline sucrose. The number of endonuclease sensitive sites decreased as the time between UV irradiation and pulse-labeling of daughter DNA increased. Further, there were significantly less endonuclease sensitive sites in the daughter DNA from cells irradiated in the G_1-phase than in the S-phase. These data indicate that very few, if any, dimers are transferred from parental DNA to daughter DNA and that the dimers detected in daughter DNA may be due to the irradiation of replicating daughter DNA before labeling.

The DNA synthesized immediately following UV-irradiatiation of mammalian cells is smaller in size than DNA from unirradiated cells (1). Eventually this small DNA is chased into higher molecular-weight DNA resembling in size parental DNA by a process referred to as postreplication repair. Some data (2,3)suggests that the replication machinery bypasses the dimer in the parental DNA strand, while other data (4) suggest that gaps are left in daughter DNA following UV-irradiation. Although the molecular events of postreplication repair are not completely understood, it is probable that, at the very least, both the by-pass and gap filling mechanisms are involved.

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Some controversy exists as to the process by which gaps made in daughter DNA are filled-in. Data (4) using the sedimentation of 313-photolyzed DNA containing bromouracil indicated that the gaps were filled in by de novo synthesis. Other data (5,6) using T_4 and Micrococcus luteus UV-specific endonuclease suggested that 3 to 30% of the pyrimidine dimers formed in parental DNA were transferred to daughter DNA. A complication in using asynchronous cells is that irradiation could make dimers in the daughter DNA as well as in parental DNA strands. If indeed the endonuclease-sensitive-sites detected in daughter DNA were the result of irradiating replicating daughter DNA, than these sites probably would not be observed in the daughter DNA of cells having low excision repair in: i, an asynchronous cell population pulse-labeled long times after irradiation (to prevent label from being added to the ends of irradiated replicons); and ii, a synchronous cell population irradiated in the non-synthesizing (G_1) phase of the cell cycle. Our data using excision deficient Chinese hamster cells, V79, and UV-endonuclease from M. luteus indicate that the number of endonuclease sensitive sites diminished as the time between irradiation and pulse-labeling increased and that few if any endonuclease-sensitive-sites were detected in the daughter DNA from synchronized cells irradiated in the G_1-phase.

Chinese hamster, V79, cells were grown in the Dulbecco's medium as previously described (7). In experiments using asynchronous cells (Table 1) approximately 6×10^5 cells were seeded in 5 ml of medium in 60 x 15 mm Falcon plastic tissue culture dishes and incubated with or without 0.02 µCi of [2-^{14}C]thymidine (50 Ci per mol) per ml. Cells labeled with [^{14}C]tymidine were used as unirradiated controls to determine non-specific nicking of unirradiated DNA by the endonuclease. In experiments utilizing synchronized cells (Table 2), 1.8×10^6 cells were seeded in 10 ml of medium in 75 cm^2 plastic tissue culture bottles. After 48 hours, cells in mitotisis and the G_1-phase of the cell cycle were shaken off and 3×10^5 cells were seeded in plastic tissue culture dishes. Before irradiation the medium was removed from the plate and cells were washed with 2.5 ml of sterile phosphate buffered saline (PBS). After removal of PBS cells were irradiated with 10 Jm^{-2} of 254-nm radiation. Immediately after irradiation, cells were incubated with warm medium and at times indicated in Tables 2 and 1 were pulse-labeled with 2.5 µCi (60 Ci per mmol) per ml for 3 hours or 2.0 µCi (7 Ci per mmol) per ml or 2 hours of [^{3}H-methyl]thymidine respectively. Cells were chased in nonradioactive medium for 12 hours to allow for the conversion of small DNA found immediately following UV irradiation into large (parental size) DNA. Approximately 20

endonuclease-sensitive-sites per 10^8D DNA were made in the parental DNA from asynchronous and synchronous cells by irradiation with 10 Jm^{-2} UV.

TABLE I
NUMBER OF UV-ENDONUCLEASE[1] SENSITIVE SITES IN DAUGHTER DNA[2] OF UV-IRRADIATED ASYNCHRONOUS CHINESE HAMSTER CELLS

Time Between UV-Irradiation and Pulse-Labeling[3]	Δ 1/Mn Number of UV-Endo Sites[4] per 10^8D
0.25 hr	1.47
2 hr	0.92
4 hr	0.63

[1] M. luteus endonuclease, the gift of Jay Doniger, was equivalent to pooled peaks I and II of the P 11 fraction according to the procedure of Riazuddin and Grossman (8).

[2] The DNA was extracted with phenol after lysis of 8 x 10^5 cells with 100 µg per ml Pronase and 0.2% Sarkosyl.

[3] Cells were irradiated with 10Jm^{-2} UV.

[4] The extracted DNA (100 µl) was incubated with 10 µl of the M. luteus extract for 20 min at 37°C, layered on top of a 0.2 ml solution containing 0.5 M NaCl and 5 mM EDTA on top of a 5-20% alkaline sucrose gradient (0.5 M NaCl, 0.1 M EDTA and 0.2 M NaOH). After centrifugation for 75 min at 50,000 RPM at 20°C in a SW 60 Rotor the number-average molecular weight (Mn) was determined. The number of UV-endo sensitive sites (Δ 1/Mn) was determined by Δ 1/Mn = 1/Mn (DNA treated with UV-endo)-1/Mn (DNA not treated with UV-endo).

When asynchronous cells were pulse-labeled 0.25 hours after irradiation, approximately 1.47 beaks per 10^8D DNA were produced in daughter DNA by UV-endonuclease (Table 1). A reasonable interpretation of these data is that dimers are transferred from parental to daughter DNA as a result of recombinational repair. However, since the daughter DNA was labeled shortly (0.25 hr) after UV irradiation, [^{3}H]thymidine could add onto the ends of an unlabeled replicon containing dimers. If this did occur, then the dimers detected in the daughter DNA could arise in unlabeled DNA attached to labeled DNA. In order to determine which one of or both of the above mechanisms accounts for the presence of dimers in daughter DNA, we lengthened the time between irradiation and pulse-

labeling of daughter DNA to allow for the joining together and replication of any irradiated unlabeled replicons. The results shown in Table 1 indicate that the number of endonuclease-sensitive sites decreases (1.47 to 0.63 per 10^8D) as the time between irradiation and pulse-labeling increases (0.25 to 4.0 hours). We find that less than 10% of the total number of dimers made by 10 Jm^{-2} were removed within the 4 hour time period of the experiment. Although 0.63 endonuclease-sites were still detected in cells pulse-labeled 4 hours after irradiation, these data suggest that not all of the dimers detected in daughter DNA result from the transfer from parental DNA.

To eliminate the possibility of irradiating replicating daughter DNA, we determined the presence of dimers in synchronous cells irradiated in the G_1 or S-phase of the cell cycle. Cells were irradiated 2 hours following mitotic shake-off (G_1-phase) and labeled 4 hours later in the S-phase of the cell cycle, or irradiated 6 hours after shake-off (during S-phase) and pulse-labeled 0.25 hour later. As is shown in Table 2, approximately 0.1 break per 10^8D was made by UV-endonuclease in daughter DNA from cells irradiated in G_1, while 1.2 breaks per 10^8 D were made in daughter DNA from cells irradiated in S-phase.

TABLE 2
NUMBER OF UV-ENDONUCLEASE SENSITIVE SITES IN DAUGHTER DNA OF UV-IRRADIATED SYNCRONIZED CHINESE HAMSTER CELLS

Hours After Mitotic Shake-Off[1]	Δ 1/Mn Number of UV-Endo Sites[2] per 10^8D
2	0.1
6	1.2

[1]Cells were irradiated or not irradiated with 10 Jm^{-2} UV either 2 or 6 hr following mitotic shake-off. At 6.3 hr after shake-off cells were pulse-labeled with [^{3}H]thymidine.

[2]Described in Table 1.

We conclude that in Chinese hamster cells: a) few if any dimers are transferred from parental to daughter DNA strands by a recombination repair process; and b) the apparent endonuclease sensitive sites that are detected in labeled daughter DNA of asynchronous cells are probably the result of adding label onto the ends of irridiated unlabeled replicons.

ACKNOWLEDGMENTS

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THE MECHANISM OF POST-REPLICATION REPAIR IN MAMMALIAN CELLS.

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INTRODUCTION

Two mechanisms have been proposed to explain post-replication repair in mammalian cells (Figure 1). The "gapped

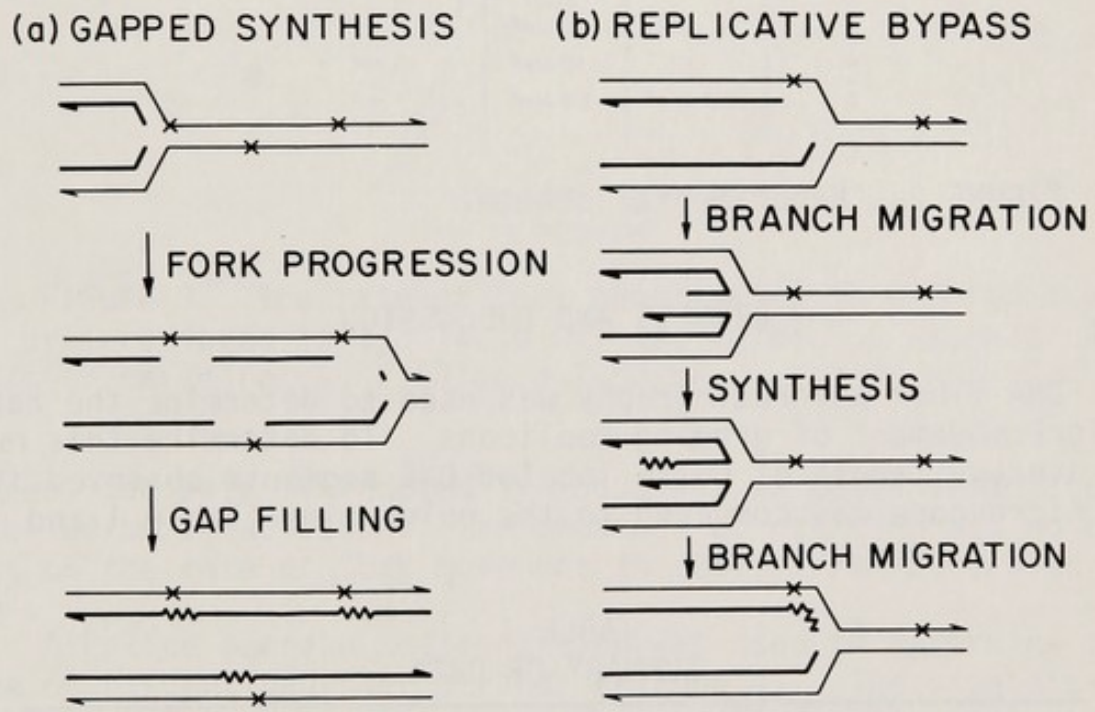

FIGURE 1. Models of post-replication repair.

synthesis" model (1) predicts that daughter strands replicated on damaged templates will contain discontinuities. The size of the nascent strands should be equal to the distance between the damages. Moreover, the rate of fork movement should be independent of the rate of discontinuity repair. The "replicative bypass" model (2,3) predicts that there will be no discontinuities in the daughter strands. Blocking repair would prevent fork movement. The experiments reported here were performed in order to distinguish between these two mechanisms.

Post-replication repair in Chinese hamster V79 cells

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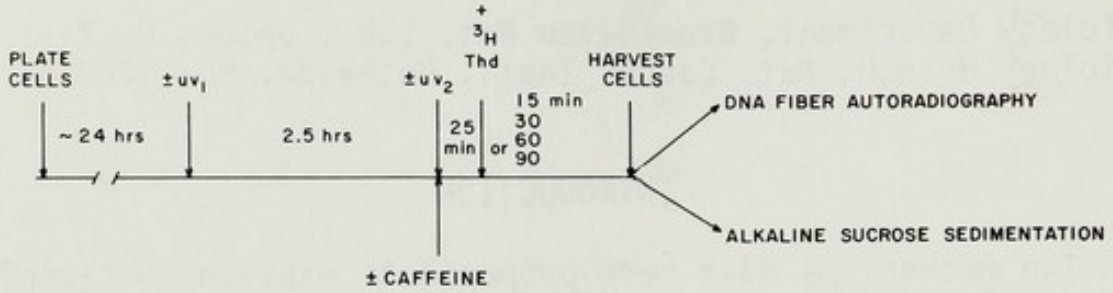
irradiated with ultraviolet light at 254 nm was examined following the experimental scheme shown in Figure 2.



EXPERIMENT	uv_1	uv_2	CAFFEINE
1	-	-	-
2	-	5 J/m²	-
3	-	10 J/m²	-
4	-	10 J/m²	1 mM
5	2.5 J/m²	7.5 J/m²	-

FIGURE 2. Experimental scheme.

RESULTS AND DISCUSSION

DNA fiber autoradiography was used to determine the rate of fork movement of growing replicons. To determine this rate, the average length of pulse labeled DNA segments observed in the microscope was compared to the pulse time (Table 1 and

TABLE I
SUMMARY OF DATA

Experiment No.	Conditions	Relative Rate of Replication	Rate of Fork Movement μm/min	15' Pulse Mode Molecular Weight x 10^{-6}	Measured Daltons/Dimer x 10^{-6}
1	0 uv	1.0	0.36	20.7	---
2	5 J/m²	0.53	0.33	10.5	8-12
3	10 J/m²	0.27	0.21	6.6	4-6
4	10 J/m² +caffeine	0.24	0.22	6.4	4-6
5	2.5 J/m² +7.5 J/m²	0.12	0.30	8.6	4-6

1 μm=10^6 Daltons.

Figure 3). It can be seen that the rate of fork movement is the same in cells irradiated with $5J/m^2$ as in unirradiated cells

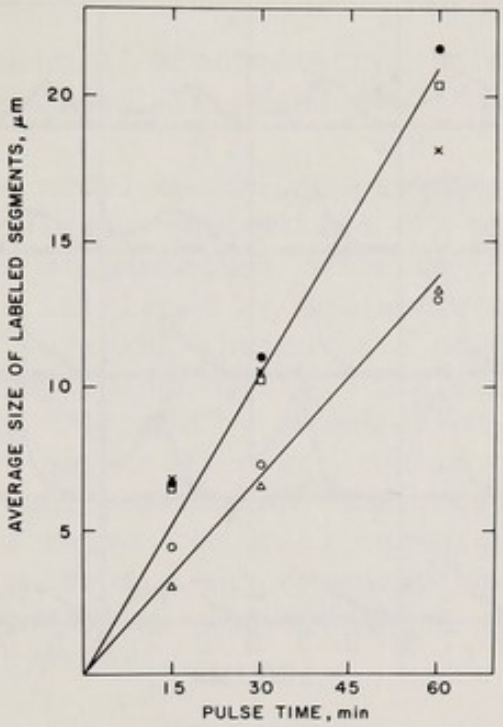


FIGURE 3. The rate of fork progression in unirradiated and uv irradiated cells. ●, o uv; □, $5J/m^2$; o, $10J/m^2$; △, $10J/m^2$ + caffeine; x, $2.5J/m^2$ + $7.5J/m^2$.

whereas the rate is reduced by about 40% in cells irradiated with $10J/m^2$ irradiation. The presence of caffeine had no effect on the rate of fork movement in cells irradiated with 10 J/m^2.

Alkaline sucrose sedimentation was used to determine the size of nascent daughter strands (Figure 4). The size distribution of nascent strands in unirradiated cells, pulse labeled for 15 or 30 min, was comparable to the size of growing replicons measured in the DNA fiber autoradiograms. After 5 or $10J/m^2$ irradiation, the size distribution of nascent strands was much smaller. The molecular weight at early pulse times is about equal to the distance between pyrimidine dimers (the major uv photoproduct) in the parental strands (Table 1). With increasing pulse times, there was increasing amounts of label in large molecular weight DNA indicating post-replication repair. There was less label in molecules $>5 \times 10^7$ daltons from irradiated cells incubated in the presence of caffeine than from cells incubated in its absence. Therefore caffeine slowed the rate of post-replication repair.

These results support the "gapped synthesis" model because

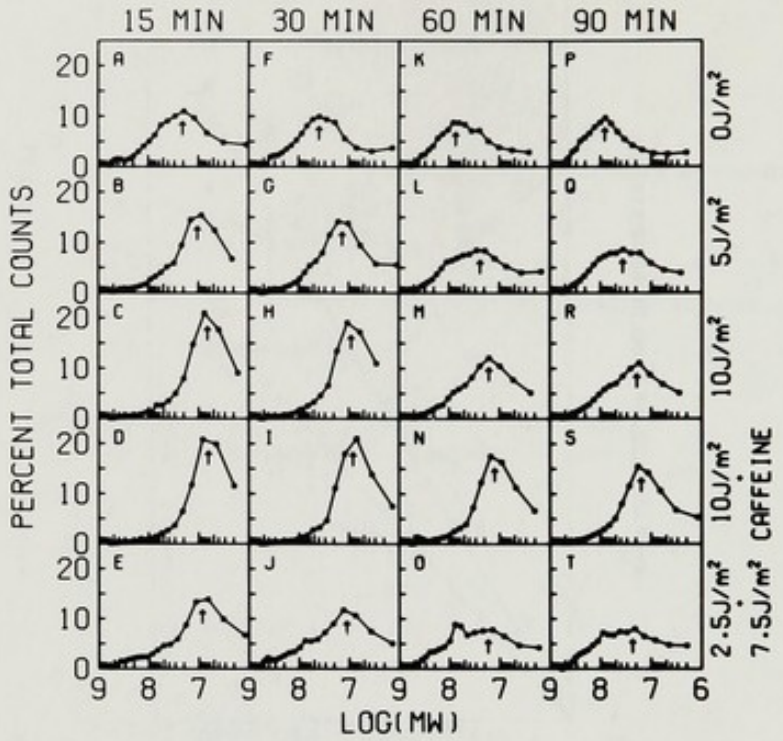


FIGURE 4. Alkaline sucrose sedimentation profiles for pulse labeled cells. The $\log_{10}$(MW) is calculated for each fraction and plotted versus the percent of total radioactivity (greater than 10^4 cpm) in each gradient. The arrows indicate the mode molecular weights.

the size of growing replicons in cells irradiated with $5J/m^2$ is the same as in unirradiated cells, while the size of nascent strands are smaller and approximately equal to the distance between uv induced lesions. Furthermore, the results show that caffeine slows down post-replication repair but does not affect the rate of fork movement.

ACKNOWLEDGMENTS

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A MODEL FOR POSTREPLICATION REPAIR OF UV DAMAGE IN MAMMALIAN CELLS[1]

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ABSTRACT The model described provides a mechanism for gap-filling in newly synthesized DNA at short times after exposure of mammalian cells to UV irradiation. The process is initiated by endonucleolytic cleavage of the undamaged parental strand at a point opposite the site of interrupted synthesis. Transient crossing-over of DNA strands, followed by movement of the linkage point, leads to gap-filling and return of the DNA strands to their original positions. The model provides for the involvement of recombinational exchange in postreplication repair and can also be applied to a mechanism in which the damaged sites act as an absolute block of DNA synthesis.

INTRODUCTION

DNA synthesized shortly after UV irradiation in a variety of mammalian cells is of lower molecular weight than in unirradiated cells (1-4). Elongation of these newly synthesized fragments proceeds largely by *de novo* synthesis (2,5). In L cells at comparable UV doses the size of newly synthesized DNA after irradiation is approximately the same as that in untreated cells (6). The major mechanism for postreplication repair in bacteria is carried out as recombination repair (7) but this mechanism occurs only at a low level in mammalian cells (8,9).

While it is accepted that UV induced damage in DNA acts to block DNA synthesis it is still not clear whether gaps are formed in DNA or whether an absolute block of synthesis occurs at the growing point (10,12). A number of models have been proposed for postreplication repair of UV damage in mammalian cells (13-15).

The model described here has some features in common with other models which invoke strand displacement in postreplication repair (13,14) and a more recent copy-choice model (M. Green, personal communication).

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MECHANISM

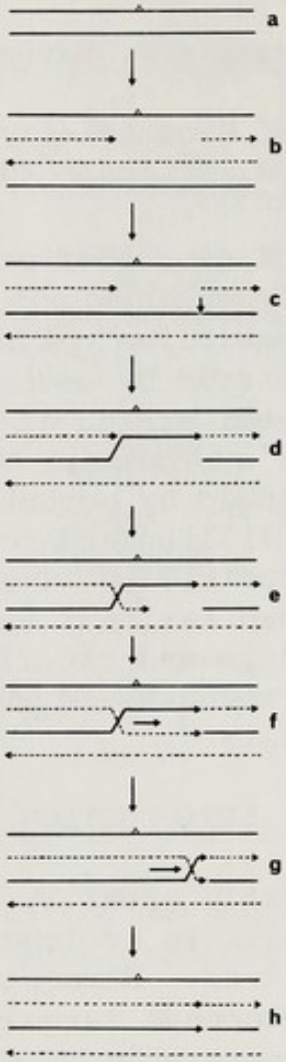

FIGURE 1. Mechanism for postreplication repair of UV damage in mammalian cells.

A series of steps are envisaged in the model presented here (Fig.1).

(a) UV gives rise to damage in DNA.
(b) Discontinuities in newly synthesized DNA occur opposite damaged sites after DNA replication.
(c) A nick is introduced in the complementary parental strand opposite the discontinuity.
(d) The nicked strand switches to pair with the damaged parental strand.
(e) Reciprocal crossing-over follows.
(f) The linkage point migrates to the right.
(g) Restoration of the DNA strands to their original positions occurs.
(h) The process of gap-filling is terminated by ligation of

the strands.

In the mechanism described here a polymerase to read past damaged sites is not required. The initial event requires the introduction of a single strand break into the undamaged parental strand at a point opposite the gap. It is postulated that this nick is similar to that observed in the case of untwisting enzyme which is not a substrate for DNA ligase (16). The introduction of such a break would not be directed at a particular base sequence but rather at a site created by the close alignment of the homologous region of the undamaged parental strand with the single stranded region containing the damaged site. A number of mammalian enzymes have been described which could provide this specificity (17-19). In addition evidence exists for an essential role for endonucleases in genetic recombination in eukaryote cells (20,21).

The presence of this nick would allow strand migration similar to that proposed to account for intermediate structures in genetic recombination. Rotary diffusion of the exchange point along the DNA molecule would allow a re-pairing of the parental strands in the region of the damaged site. This step is followed by reciprocal crossing over at the point of interruption of DNA synthesis followed by polymerization. Movement of the linkage point (22) to the right by rotary diffusion restores the DNA strands to their original positions. This would then allow ligation of the strands. The model described here is consistent with the observation that exchange of pyrimidine dimers occurs between

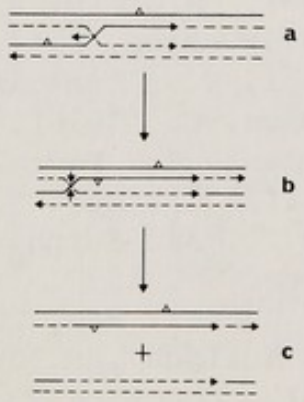

FIGURE 2. Transfer of pyrimidine dimers between parental and daughter strands by recombination.

parental and daughter strands after UV irradiation (23). Strand breakage at the crossing over point or as the point of linkage moves to the left would result in such transfer (Fig. 2).

The intermediate structures depicted in this model (Fig.1) are consistent with results from density gradient analysis (13), electron microscopy (14), photoreversal of BUdR-substituted regions (2,24), and enzymological studies (9).

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THE KINETICS OF POSTREPLICATION REPAIR IN MAMMALIAN CELLS AS STUDIED BY THE ALKALINE ELUTION TECHNIQUE[1]

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ABSTRACT The alkaline elution technique was used to study the kinetics of postreplication repair in Chinese hamster cells. The validity of this approach was tested by confirming several known aspects of this repair mode. Further investigations showed that the ability to synthesize normal-sized DNA at late times after exposure to ultraviolet light could not be inhibited by cycloheximide.

INTRODUCTION

The alkaline elution technique developed by Kohn et al. (1) has been used to study postreplication repair (PRR) in cultured Chinese hamster cells. In this method, the cells are lysed on polyvinylchloride filters and the DNA is then slowly eluted through the filters with EDTA-tetrapropylammonium hydroxide (pH 12.1). The rate of elution of the DNA increases with decreasing single-strand length. Although this technique is more qualitative than quantitative in that it does not yield molecular-weight values, it does have several advantages over alkaline sucrose gradients. It is simple to use and several filters can be eluted simultaneously in a relatively short time. Furthermore, results in the form of alkaline elution profiles are easily interpreted. The validity of the alkaline elution technique for use in studies of PRR was tested by confirming several known aspects of this repair mode. This method has been used subsequently to characterize the ability of mammalian cells to synthesize normal-sized DNA at late times after exposure to ultraviolet light (UVL). The dependency of this ability upon de novo protein synthesis was also investigated.

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METHODS

Chinese hamster ovary (CHO) cells in monolayer culture were used in these experiments. The cells were cultured in McCoy's 5A medium supplemented with 20% fetal calf serum. All incubations were at 37°C in a 5% CO_2 atmosphere.

In a typical experiment, several 60 mm petri dishes were seeded with $1x10^6$ CHO cells and incubated overnight. Following irradiation with UVL, the cells were incubated for various periods of time in complete medium prior to pulse labeling for 15 min with 2μCi/ml ^{3}H-thymidine (^{3}H-TdR) plus .25 μg/ml cold TdR. 2.5 μg/ml cold TdR was also added to the medium in experiments requiring incubation after pulse labeling.

The alkaline elution method employed was essentially that described by Kohn et al. (1). Briefly, the cells were removed from the petri dishes with trypsin, diluted with cold phosphate buffered saline, and impinged on 25 mm diameter, 2 μm pore size polyvinylchloride filters. The cells were then lysed with 0.2% Sarkosyl. The remaining DNA was rinsed with .02M EDTA (pH 10.3) and eluted with 0.1M tetrapropylammonium hydroxide, 0.02M EDTA (pH 12.1). The rate of elution was .15 ml/min and 10 fractions were collected directly into scintillation vials over a 2 hr period. The ^{3}H-radioactivity remaining on the filter and in each fraction was measured in a liquid scintillation counter. The fraction of DNA retained on the filter as a function of fraction number was then calculated.

RESULTS

Although the alkaline elution technique had been previously shown to be applicable to pulse-labeled DNA (1), the validity of this method for use in studies of PRR was tested by confirming several known aspects of this repair mode. The results of one such test are shown in figure 1. In this experiment, cells were irradiated with 50 ergs/mm^2 of UVL, incubated for 30 min, and then pulse labeled for 15 min. The alkaline elution profiles (fig.1) show that the size of the DNA synthesized in the irradiated cells is smaller than that synthesized in comparable control cells. If, however, the irradiated cells are incubated for an additional 60 min following pulse labeling, the size of the DNA increases to then nearly equal the size of the DNA of the control. The size of the DNA in control cells also increases during a 60 min incubation following pulse labeling (fig. 1).

These results are qualitatively similar to the previous findings of a number of investigators (2,3,4) which have been

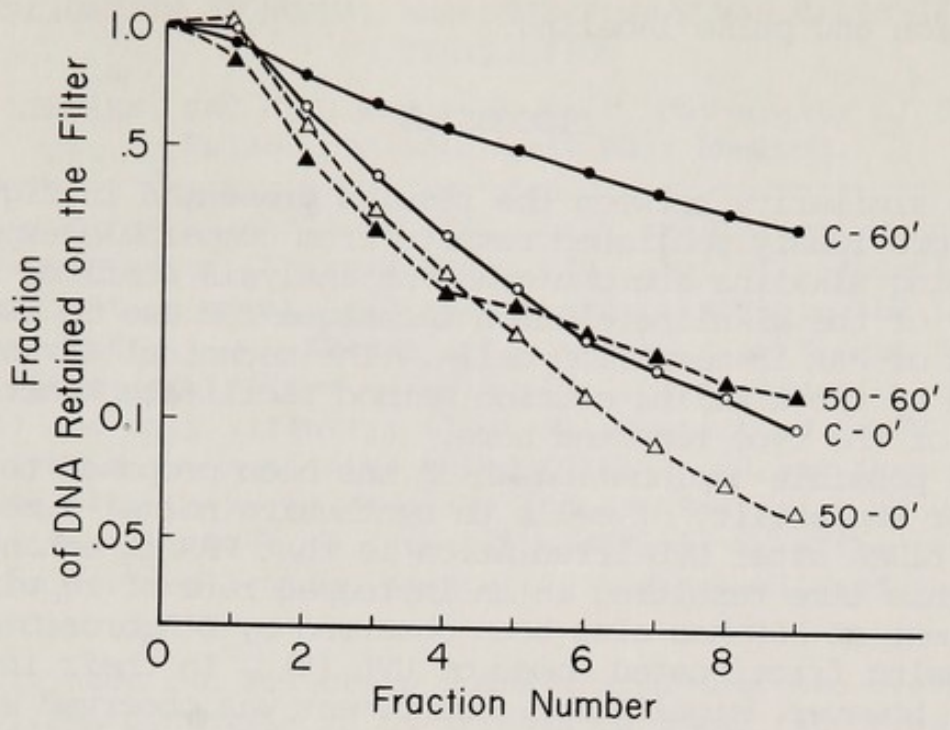

Figure 1. Alkaline elution profiles of DNA synthesized in UVL-irradiated CHO cells. The cells were irradiated with 50 ergs/mm^2 of UVL, pulse labeled for 15 min with ^{3}H-TdR, and either analyzed immediately (△---△) or incubated for an additional 60 min in medium without label (▲---▲) prior to analysis by alkaline elution. The profiles for DNA from unirradiated control cells labeled in a similar manner are also shown. o---o, pulse labeled only; •---•, pulse labeled and incubated for 60 min.

interpreted to indicate that DNA synthesis is interrupted at the site of photoproducts in DNA and that these interruptions (or gaps) are sealed within a short time. This interpretation of such observations forms the basis for most current models of PRR in mammalian cells (5).

Another aspect of PRR which has been investigated using the alkaline elution method is the recovery of the ability to synthesize normal-size DNA at long times after UVL irradiation (3,6,7). Typical results (not shown) indicate that the alkaline elution profile of DNA from cells which have received 50 ergs/mm^2 and then pulse labeled at 6 hours after irradiation is similar to that of unirradiated cells. An analysis of the kinetics of this return to normal synthesis characteristics showed that this recovery was not evident within either 2 or 4 hours. Further experiments, using cycloheximide, demonstrated that the ability to make normal-size DNA was not dependent upon the synthesis of new proteins during the 6 hours between

irradiation and pulse labeling.

DISCUSSION

The similarity between the results presented in figure 1 and the previously published results from comparable experiments using alkaline sucrose gradient analysis confirms the validity of the alkaline elution technique for use in investigations of PRR in mammalian cells. The technical advantages provided by the alkaline elution method facilitate kinetics studies of the type reported here.

One possible explanation which has been proposed to account for the ability of cells to synthesize normal-size DNA at late times after UVL irradiation is that PRR is enhanced during this time resulting in an increased rate of repair (6). Enhancement of PRR has also been observed by D'Ambrosio and Setlow using fractionated doses of UVL (8). In their investigations, however, significant enhancement was observed within 2 hours and it was inhibited by cycloheximide. We conclude, on the basis of our results, that if the ability to synthesize normal-size DNA at late times following UVL irradiation represents an enhancement of PRR, then this enhancement may be significantly different from that observed by D'Ambrosio and Setlow.

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DNA REPLICATION IN NORMAL AND DEFECTIVE HUMAN CELLS AFTER UV IRRADIATION

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Most xeroderma pigmentosum (XP) cells are defective in excision-repair after UV-irradiation (1), partially defective in postreplication repair (2) and very sensitive to the lethal effects of UV (1). XP variants have no significant defect in excision-repair, are defective in postreplication repair (1,2,3) and only slightly, if at all, sensitive to UV (1,4). Cells from a sun-sensitive child (11961) (5) and from patients with Cockayne syndrome (6) are sensitive to UV-irradiation but have no detectable defects in either excision (5,6) or postreplication repair (2, and unpublished observations).

Recently Rude and Friedberg demonstrated that the overall rate of DNA synthesis in UV-irradiated human fibroblasts was initially depressed but regained normal levels about six hours after irradiation (7). This recovery did not occur in excision-defective XP cells and was somewhat reduced in XP variants. Rudé and Friedberg suggested that this restoration of normal rates of DNA synthesis was correlated with cell survival and was a consequence of excision repair. We have carried out similar experiments with 11961 and Cockayne cells, but measuring DNA synthesis by thymidine incorporation into DNA, rather than by autoradiography. Our findings with normal and XP cells agree with those of Rudé and Friedberg (7). We have also found that 11961 and Cockayne cells, like XP cells, fail to recover normal rates of DNA synthesis 5-8 hours after irradiation. These observations support the suggestion (7) that this recovery is correlated with, and can be used as a useful early indicator of cell survival. On the other hand they contradict the idea (7) that it is primarily a consequence of excision repair. Since 11961 (5) and Cockayne cells (6) have normal unscheduled DNA synthesis and dimer excision, it seems unlikely that the failure to recover normal rates of DNA synthesis can be due to a lack of excision-repair.

The relationship of the restoration of normal DNA synthesis rates to the ability of mammalian cells to synthesize DNA of normal size at late times after UV (8) is unclear, since the latter has been demonstrated in all cells studied. It is possible that these recovery processes are dependent on inducible enzymes, though at present there are no data available to support this suggestion. If such inducible functions are indeed involved, there is no evidence to suggest that they are analogous to the inducible error-prone repair

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which has been intensively studied in Escherichia coli (9).

Cells from 11961 (5) and Cockayne patients (Arlett, C. F., this volume) are also hypersensitive to the killing effects of ethyl methanesulfonate (EMS). Unlike the response to UV however, EMS caused only a slight depression in the rate of DNA synthesis in normal human cells and there were no reproducible differences between normal, 11961 and Cockayne cells up to 24 hours after treatment with EMS. The cross-sensitivity of 11961 and Cockayne cells to UV and EMS suggest that a common enzymatic step is involved in the repair of damage induced in human cells by these agents. The DNA synthesis data, however, indicate that the way in which the two agents kill human cells is quite different.

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REPLICATIVE REPAIR IN XERODERMA PIGMENTOSUM (XP) VARIANTS[1]

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ABSTRACT Caffeine exerted differential effects on 12 XP variant and some XP strains after UV exposure. Its killing potentiation and replicative-repair inhibition varied so distinctly as to subgroup XP variants. Subgroups I and II with skin cancers manifested defective and caffeine-sensitive replicative repair, while Subgroup III without cancers did almost normal and caffeine-resistant repair.

INTRODUCTION

An XP variant group having normal excision, but defective postreplication repair for UV damage (1, 2) exists in addition to several excision-defective XP groups (3). As a possible mammalian postreplication repair model, replicative repair has been proposed (2, 4), and human cells exhibit at least caffeine resistant (CRRR) and its sensitive replicative repair (CSRR)(1, 2). Here I describe differential caffeine effects on and a possible subgrouping of 12 variants and a strong coincidence between CSRR manifestation in vitro and skin carcinogenesis.

MATERIALS AND METHODS

Cells and Survival Assay. 12 XP variants (Table), group A XP2OS and SV40-transformed XP2OS-SV, group D XP10TO, and normal human (NHSF6) cells were used. UV survival was assayed for clone forming ability and synergistic caffeine effect on UV killing was analyzed for Do (mean lethal UV dose) reduction ($S(Do)$) as described previously (2): $S(Do)= {}_e\text{-}C/Co$, where C and Co are caffeine dose used and 37% Do-reduction dose, respectively.

Molecular Studies. The DNA after UV irradiation (0 or 10 J/m^2), 1 h-[^{3}H]thymidine (dT) labeling (5 μCi/ml), and a 4 h-chase with or without 2 mM caffeine was centrifuged in 5-20% alkaline sucrose (2). Alternatively, cells were incubated in 1 μM BdU and 1 μM FdU for 1 h, irradiated (0 or 20 J/m^2),

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labeled for 1 h with 5 μCi/ml (1 μM) of [^{3}H]dT in 10 μM BdU and FdU, and chased for 4 h with or without 2 mM caffeine. DNA was extracted, fragmented and centrifuged in neutral CsCl (2) for replicative repair (2). Unscheduled synthesis (UDS) was measured on autoradiographs made after 4 h [^{3}H]dT label following irradiation with 20 J/m^2, as described previously (2).

RESULTS AND DISCUSSION

Effects of Caffeine on UV Survival. 12 XP variants showed 100% UDS (Table) and a little higher UV sensitivity than did normal cells. Caffeine enhanced killing of several XP variants. Potentiation can be quantitated by the 1st-order *Do* reduction (2): $S(Do) = e^{-C/Co}$ (Fig. 1, ordinate). Fig. 1 shows differential caffeine potentiation in XP13BE, XP3KO and XP13KO cells. *Co* values as in Fig. 1 divide the variants into 3 subgroups. Subgroup I (5TO, 12KO, 13BE) was potentiated most drastically (*Co* = 1.5 mM), Subgroup II (3KO, 8KO, 9KO) intermediately (*Co*= 2.7 mM), and Subgroup III (1MI, 4TO, 7KO, 13KO, 14KO, 20OS) not (*Co* = ∞), as in XP2OS and normal cells (Table) (4). Aneuploid XP2OS-SV, like many established lines (2), was most potentiated (Fig. 1), and 4TO and 5TO variant sisters fall into separate III and I, suggesting that CRRR can be transformed to CSRR.

Molecular Studies of Replicative Repair. To substantiate the above characteristic findings, molecular effects of a 2-mM caffeine concentration were studied. Weight average molecular weight (Mw) of 10 J/m^2-irradiated, 1 h-labeled and 4 h-chased DNA was determined for each strain by an alkaline sucrose technique (2). I calculated Mw ratios of XP variants and XPs to normal (ratio = 1). Table shows the quantitative results indicating the Subgroup-specific defect and caffeine sensitivity. Corresponding well with the survival data (Fig. 1, Table), Subgroup I manifests the slowest and most caffeine-sensitive repair (CSRR), as judged from near-minimum ratios. In Mw rises during chase, Subgroup II exhibits intermediate defect and

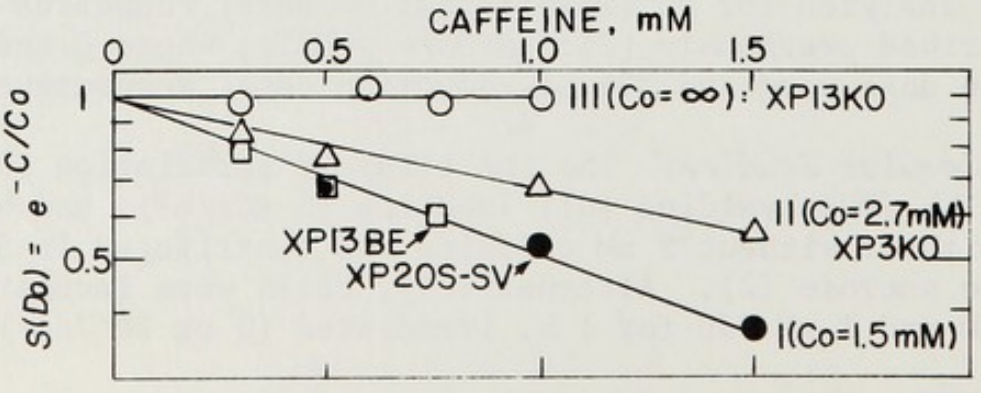

FIGURE 1. Differential caffeine effect on Do reduction.

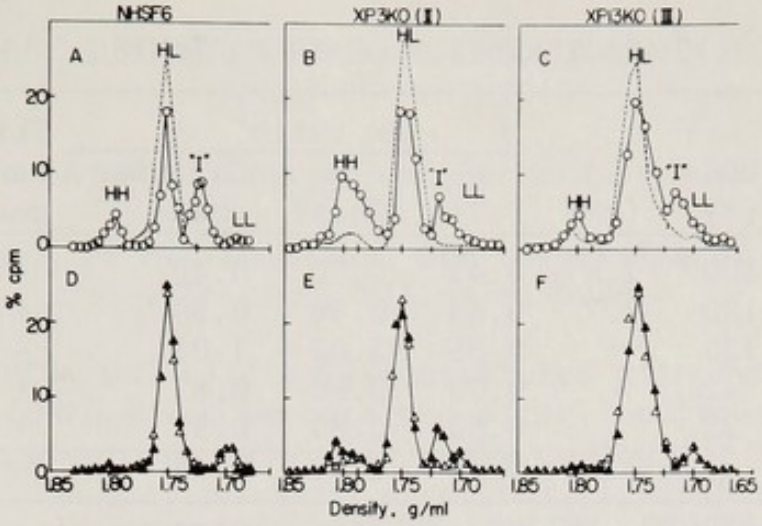

FIGURE 2. Replicative repair studied by a neutral CsCl method.

caffeine sensitivity (partially CSRR), and neither does Subgroup III (ratio = 1.03; CRRR) as in NHSF6 cells (Table). Moreover, Groups A and D XPs manifest CSRR partially (Table), while XP2OS-SV does only CSRR (Fig. 1), since caffeine abolished elongation (2), giving a minimum Mw ratio of 0.35 (Table).

As studied in XP5TO variant (2), I examined caffeine effects on replicative repair in XP3KO (II) and XP13KO (III) cells. In Figs. 2A to C (broken line), control, 1h-labeled DNA from all kinds banded exclusively at a hybrid (HL) density of 1.75 g/ml. [^{3}H]DNA replicated for 1 h on damaged templates banded at HH (1.80 g/ml) and "I" (1.72 g/ml) in addition to main HL (Figs. 2A to C, open circle). HH and "I" were converted to HL during chase without caffeine (Figs. 2D to F, open triangle). We interpret these unique features as showing that the dimer acts as temporal block to replication and branch migration may occur to copy sequences opposite the lesion by use of a newly replicated strand as an alternative template, followed by reanealing to resume normal synthesis past the dimer (2, 4). The caffeine chase partially prevented the conversion of HH and "I" to HL only in XP3KO (Fig. 2E, closed triangle), though more in the previous XP5TO (I) (2). But, no such prevention occurs in XP13KO (III) and NHSF6 in Figs. 2D and F, respectively.

Caffeine is so far known to potentiate UV killing of all XP variants that possess defective postreplication repair (1, 2, 5, 6). This is not always so as the cases increase, and here I have shown such different responses of XP variants to caffeine that make subgrouping possible. My data indicate that Subgroups I, II, and III manifest the most defective CSRR, partially defective CSRR, and almost normal CRRR, respectively. Particularly, one can find a strong coincidence between defective CSRR in the former two and 100% skin cancer incidence

TABLE
CHARACTERISTICS OF XP VARIANTS

Group or Subgroup[a]	UDS (%)	Co (mM)	Mw ratio[b] Pulse	Chase	Caffeine chase	Skin cancers	CSRR
Variant I	100	1.5	0.41	0.70	0.40	+	+++
Variant II	100	2.7	0.68	0.86	0.59	+	++
Variant III	100	∞	0.85	1.03	1.03	-	-
A (XP20S)	<2	∞	0.70	0.90	0.81	+	+
D (XP10TO)	25	-	0.60	0.80	0.57	+	++
XP20S-SV	<2	1.5	0.98	1.00	0.35		

[a]Subgroup I: XP5TO, XP12KO, XP13BE; Subgroup II: XP3KO, XP8-KO, XP9KO; Subgroup III: XP1MI,XP4TO, XP7KO, XP13KO, XP14KO, XP20OS (our unpublished data)

[b]Alkali-sucrose Mws for pulsed, chased, and caffeine-chased DNA were 170, 258, and 254 X 10^6, respectively, in exposed NHSF6.

(Table). Of interest is that UV induces more mutations in CSRR-showing XP13BE variant than in normal (6). Subgroup III patients manifesting only CRRR had no cancers yet (100%) (Table), being reasonable in a view that these cells appear normal in both excision and replicative repair. Slight CSRR is shown in some XP groups (5)(Table) with a highly carcinogenic potential.

ACKNOWLEDGMENTS

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DNA BIFILARLY SUBSTITUTED WITH BROMODEOXYURIDINE IN THE FIRST ROUND OF SYNTHESIS[1]

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ABSTRACT DNA bifilarly substituted with bromodeoxyuridine (DNA_{HH}) can be obtained after incubation of human lymphoblastoid cells with heavy analog for short periods. DNA_{HH} is produced after cell lysis but originates from growing points and provides an assay for the proportion of label incorporated near regions of DNA synthesis. DNA_{HH} accumulated when excision defective lymphoblastoid cells were incubated with caffeine or chemical mutagens. The accumulation of DNA_{HH} was accompanied by the synthesis of shortened pieces of daughter DNA.

INTRODUCTION

Mammalian cells are able to replicate past DNA lesions by a non-recombinational mechanism (1). A possible mechanism suggested to account for this bypass postulates a single strand gap in daughter DNA synthesis starting at the site of the lesion which is later "filled in". A second hypothesis supposes that DNA synthesis is held up at the site of the lesion and that as a result of the single strandedness present at DNA growing points (2) strand displacement and branch migration occur, providing a daughter strand which can serve as a template for synthesis (3).

The second hypothesis predicts that cells treated with chemical mutagens and incubated in bromodeoxyuridine (BrdU) containing medium should accumulate DNA bifilarly substituted with analog (DNA_{HH}) in the first round of synthesis (3). However, the very large proportion of this material observed made it unlikely that most DNA_{HH} originated *in vivo*. We have recently investigated the mode of origin of DNA_{HH} and shown that much of it originated by branch migration after cell lysis (4). The purpose of this paper is to summarize this evidence, to show that measurement of DNA_{HH} is a useful probe for events at the DNA growing point, and to present evidence supporting the view that bypass of lesions is an event occurring at DNA growing points.

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2. Fellow of the Leukemia Society of America

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RESULTS and DISCUSSION

Up to 25% of the radioactivity incorporated was observed in DNA_{HH} when a lymphoblastoid line of excision defective xeroderma pxigmentosum cells (XPA-3; (5) treated with acetoxy acetyl aminofluorene (AAAF) or with anti benzpyrene diolepoxide (BPDE) was incubated with BrdU for approximately one hour. In addition, CsCl gradients also contain peaks at hybrid density and at an intermediate density of about 1.71 g/cm^3. This DNA_{int} fraction includes fragments derived from the DNA growing point (6). Shearing DNA_{int} yields DNA_{HH} and hybrid DNA, indicating the origin of DNA_{HH} from growing points (4). DNA_{HH} is "chased" into the DNA hybrid region on incubation (6). Since treatment of XPA-3 cells with trioxsalen and near UV light prior to cell lysis reduced the yield of DNA_{HH} below detectable limits (Fig. 1) we concluded (4) that DNA_{HH} was formed from growing points after cell lysis although the formation of DNA_{HH} *in vivo* in pieces smaller than the distance between trioxsalen cross links and not susceptible to shear has not been excluded.

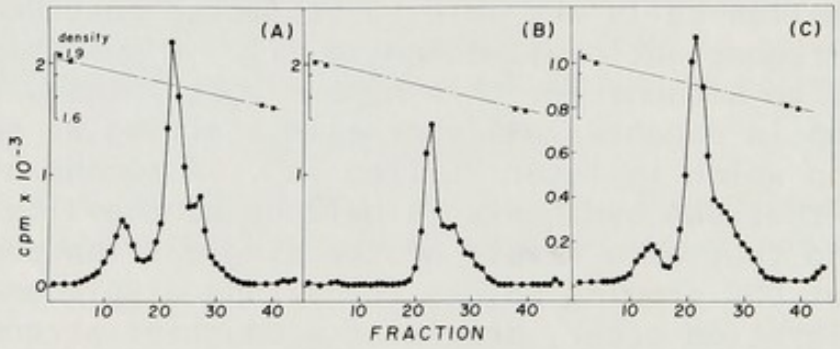

Fig. 1

CsCl gradients of DNA from cells: A, not cross linked; B, Treated with trioxsalen and near UV immediately before lysis; C, Treated with trioxsalen and UV after lysis.

In order to demonstrate the usefulness of measurements of DNA_{HH} as a probe for events at the DNA growing point we treated cells with 10mM caffeine which inhibited DNA synthesis to the 50% level as measured by ^{3}HdT incorporation. The labeling time for caffeine treated cells was adjusted so that treated and control cells incorporated equivalent amounts of isotope. DNA_{HH} was isolated by centrifugation through a CsCl gradient after a standard extraction protocol which included shearing of DNA by ten passages through a 26G needle followed by 60 min reaction with *N. crassa* endonuclease (4). Both the absolute and relative amounts of DNA_{HH} were in-

TABLE I

EFFECT OF CAFFEINE ON YIELD OF DNA_{HH} AND ON THE SIZE OF NASCENT DNA IN ALKALINE SUCROSE GRADIENTS

Concentration of caffeine (mM)	Labelling time (min)	Overall incorporation of ^{3}H-dT (cpm/µg of total DNA)	Incorporation into HH region cpm/µg of total DNA	Relative yield of DNA_{HH} (%)	Mol. wt. of nascent DNA[a] x10^{-7} daltons)
0	10	690	102	14.8	11.2
	20	1479	115	7.8	13.1
10	20	833	198	25.7	1.97
	40	1255	254	24.9	5.83

[a]XPA-3 cells were preincubated with caffeine for 30 min before labelling with ^{3}H-dT (66 Ci/mmol; 30 µCi/ml) in the presence of BrdUrd (3.3×10^{-5}M) and FdUrd (10^{-6}M). Weight-average molecular weights were calculated from sedimentation profiles of DNA in alkaline sucrose gradients.

creased in XPA3 cultures treated with caffeine (Table 1). At least two possible explanations account for the increased amount of DNA_{HH} in caffeine treated cultures: a) the length of displaced strands is longer; and b) elongation is depressed and more BrdU remains in the vicinity of the growing point leading to an accumulation of DNA_{HH} (Fig. 2A). The observation (Table 1) that the molecular weight of the nascent DNA in

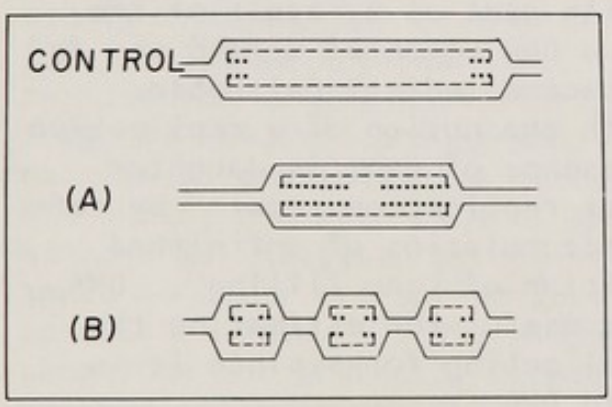

Fig. 2A

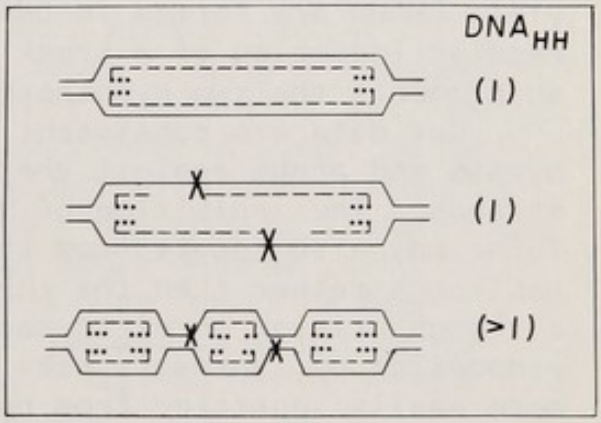

Fig. 2B

alkaline sucrose is much smaller in caffeine-treated than in control cells and the finding that the M_n of DNA_{HH} in neutral sucrose was 2.7×10^6 for caffeine treated cultures and 5.3×10^6 for controls favors the latter hypothesis and confirms an earlier conclusion by Lehman (7).

Incubation of XPA3 cells with concentrations of AAAF or BPDE sufficient to inhibit DNA synthesis to the 50% level also resulted in an increase in the yield of DNA_{HH} (Table 2). The yield of DNA_{HH} was inversely related to the size of the daughter DNA produced. The lower molecular weight of DNA synthesized after treatment with AAAF or BPDE could be accounted

TABLE II

Effect of Chemical Mutagen Treatment on the Yield of DNA_{HH}

Treatment	Dose (μM)	Rate of DNA synthesis (% of control)	Labelling time (min)	Relative yield of DNA_{HH} (%)	Mol. wt. of nascent DNA ($x10^7$ dalton)
Control XPA-3	----	100	20	5.6	12.7
BPDE	0.5	51.5	40	9.2	7.4
AAAF	7.0	53.5	40	15.8	4.3

for as the result of gaps left in the daughter strand (1) or as the synthesis of DNA in shortened replicating units resulting from a temporary block to synthesis. However, the increased yield of DNA_{HH} accompanying the shortened pieces of newly synthesized DNA is most easily accounted for by supposing that the block does indeed occur at growing points and that DNA synthesis in these XPA3 cells is held up at the point of the lesion (Fig.2B). This conclusion depends, of course, on the supposition that "gapped DNA" would not result in the formation of DNA_{HH}. Models can be drawn (see Lavin, this volume) in which replicating-like structures which would yield DNA_{HH} are formed in DNA with gaps as a result of the regular induction of a break in a DNA parental strand and in such models the two hypotheses become indistinguishable.

Our data are consistent with the notion of a replicative bypass and argue against the presence of gaps in daughter strands. The inhibition of "post replication repair" by caffeine may also result from the accumulation of unfinished replicons rather than the inhibition of "gap filling". DNA_{HH} although arising *in vitro* can be useful for estimating the proportion of synthesis near replicating forks since it is more easily separated from hybrid DNA.

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DNA TEMPLATE BREAKAGE AND DECREASED EXCISION OF HYDROCARBON DERIVED ADDUCTS FROM CHINESE HAMSTER CELL DNA FOLLOWING CAFFEINE-INDUCED INHIBITION OF POST REPLICATION REPAIR

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ABSTRACT Incubation in the presence of caffeine of Chinese hamster cells that have been treated with either 7-bromomethylbenz(a)anthracene (7-BMBA) or 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-benzo(a)pyrene diol epoxide, ADE) results in the formation of low molecular weight nascent DNA as a consequence of inhibition of post replication repair. In addition caffeine also induces the slow production of breaks in the template DNA of 7-BMBA and ADE-treated cells and decreased excision of 7-BMBA-bound adducts from DNA.

INTRODUCTION

7-Bromomethylbenz(a)anthracene-bound products are excised slowly from the DNA of both Hela and Chinese hamster cells (1). Following treatment with low doses of 7-BMBA complete replication of DNA can occur prior to excision of all lesions from the DNA, which suggests the existence of some, as yet undefined, mechanism for circumventing unexcised lesions in DNA during DNA replication. However, excision of 7-BMBA-bound adducts is inhibited by concentrations of 7-BMBA that, while toxic to cells are not so great that they would be expected to inhibit enzymes (1). The mechanism for this inhibition is therefore not presently understood. Incubation of 7-BMBA treated cells in the presence of a non toxic concentration of caffeine results in an enhancement of both toxicity and chromosome damage (2). Moreover caffeine reverses the 7-BMBA induced depression in the rate of DNA synthesis but at the same time inhibits ligation of newly-synthesised DNA molecules (2). Since the size of the nascent DNA molecules approximates to the spacing between 7-BMBA bound products in DNA it was proposed that, in the presence of caffeine, DNA replication cannot proceed past lesions in the template strand of DNA and therefore gaps, presumably opposite these lesions, are left in the nascent DNA. We have therefore speculated that the presence of these gaps in nascent DNA would interfere with steps in the excision repair of adducts in the DNA. We

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now report that during incubation in the presence of caffeine of hydrocarbon-treated cells breaks are formed in template DNA. In addition, in the presence of caffeine there is decreased excision of 7-BMBA bound products from DNA. We also show that damage introduced into DNA by ADE is similarly modified in the presence of caffeine.

MATERIALS AND METHODS

Conditions for growing Chinese hamster cells in stirred suspension culture, treating them with 7-BMBA, labelling template and nascent DNA with [^{14}C]TdR and [^{3}H]TdR, respectively and measuring the sizes of these labelled DNAs by sucrose gradient sedimentation have been described (2,3). Anti benz-(a)pyrene diol epoxide (kindly supplied by Dr. Harvey) was administered in dimethylsulphoxide solution. [^{14}C]TdR pre-labelled Chinese hamster cells were treated with ^{3}H-labelled 7-BMBA (1) and the change in the ^{3}H/^{14}C ratio permitted a calculation of loss of 7-BMBA adducts from DNA during post treatment incubation in the presence and absence of caffeine.

RESULTS

Caffeine Induced Inhibition of Ligation of Nascent DNA in ADE Treated Cells. Incubation of ADE-treated cells in the presence of caffeine reversed the ADE-induced depression in the rate of DNA synthesis. However despite this increased rate of DNA synthesis the size of nascent DNA synthesised during 4 hours after treatment with ADE in the presence of caffeine is reduced in a dose-dependent manner relative to that in control or ADE only-treated cells, fig. 1. Moreover a comparison of the size of the nascent DNA molecules with the extent of reaction with DNA after a given dose of ADE (data kindly supplied by Dr. R. Newbold) suggests that most ADE-induced lesions (like 7-BMBA induced adducts (1)) are recognized by a caffeine-sensitive post replication repair process. Some ligation of DNA molecules occurs slowly during further incubation in caffeine after treatment with low doses of ADE.

Sedimentation Profiles of Template DNA in Hydrocarbon-treated Chinese Hamster Cells During Incubation in the Presence or Absence of Caffeine. The template DNAs in cells treated with either 7-BMBA or ADE and then incubated for 4 hours in the presence of caffeine were all essentially the same size (approx 4 x 10^{8} daltons molecular weight) as the DNAs from untreated or caffeine-only treated cells. Neither agent therefore induces any immediate breaks or alkali-labile sites into DNA of this size. Moreover, following a further 20 hours incubation of cells in the presence of caffeine,

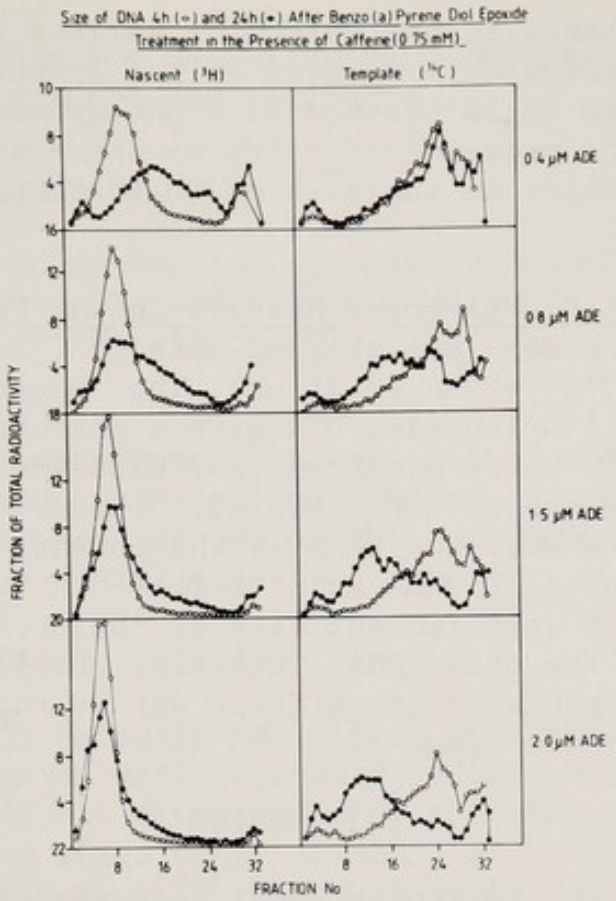

Figure 1.

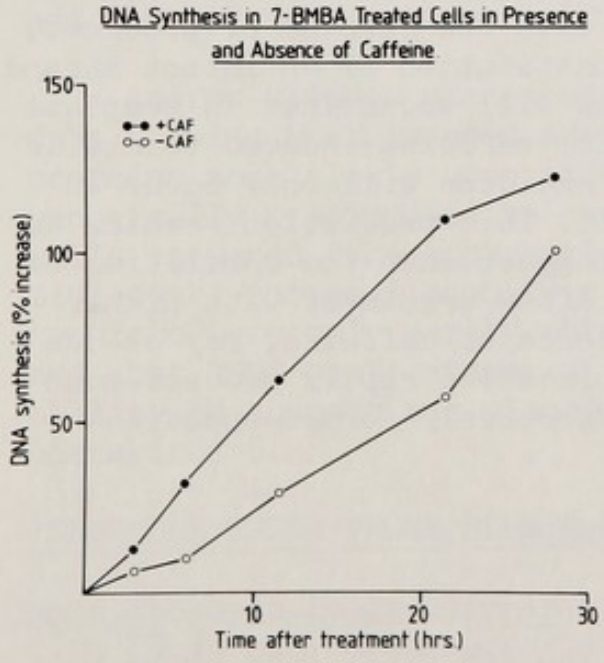

Figure 2.

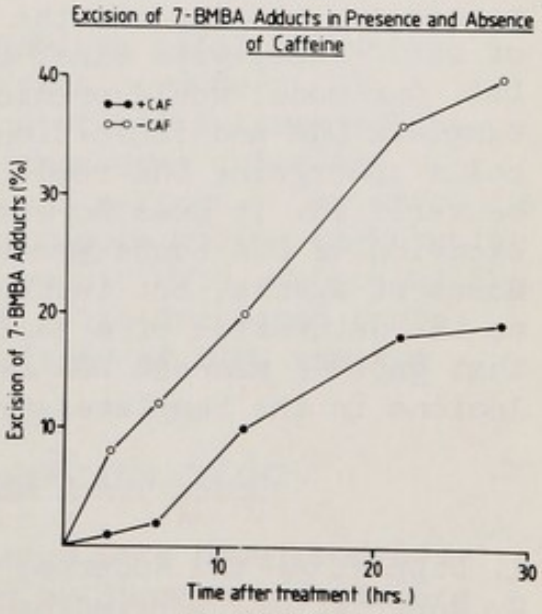

Figure 3.

there was no change in the molecular weights of the template DNAs in hydrocarbon-only treated cells. By contrast, we observed that in cells treated with the hydrocarbons and then incubated in the presence of caffeine there was a marked dose-dependent reduction in the size of their template DNAs (3) fig. 1.

Excision of 7-BMBA Bound Adducts in the Presence of Caffeine. Cells were prelabelled with [^{14}C]TdR prior to treatment with [^{3}H]7-BMBA (0.25 μM). The change in the specific activity of ^{14}C-labelled DNA gave a measure of the amount of DNA synthesis which occurred in 7-BMBA treated cells in the presence or absence of caffeine. There was more DNA synthesis in the presence than in the absence of caffeine fig. 2. The loss of 7-BMBA adducts from DNA was obtained from the decrease in specific activity of ^{3}H-labelled DNA, after correction for the above DNA synthesis. Excision of 7-BMBA adducts in the presence of caffeine was approximately 50% of that in its absence fig. 3.

DISCUSSION

We propose the following model to explain the above effects of post treatment incubation of hydrocarbon-treated cells in the presence of caffeine. The caffeine-induced gaps in nascent DNA probably do not interfere with the incision step of an excision repair process. However it can be envisaged that the absence of a suitable template is likely to block other steps in the repair process, such as the exonucleolytic degradation of DNA with the removal of products, repair replication and the reconstitution of an intact strand of DNA. Hence, with time, breaks will accumulate in template DNA. Our model would predict that caffeine-induced breaks in template DNA and inhibition of excision will only occur in cells undergoing DNA replication. This prediction remains to be verified. It does moreover suggest that the inhibition of excision of DNA bound products after treatment with higher doses of agents, but in the absence of caffeine, may be due to the saturation of a post replication repair process such that gaps in nascent DNA are effectively formed opposite lesions in the template strand.

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VIRAL PROBES FOR MAMMALIAN CELL DNA REPAIR: RESULTS AND PROSPECTS

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In this summary I shall point to the reports given in the workshop, and emphasize new findings I consider interesting, rather than review the workshop presentations which are already fairly short.

WORKSHOP REPORTS:

Sarasin and Hanawalt as well as Williams and Cleaver reported studies on various repair associated changes measured in SV40 DNA extracted from infected monkey cells at various times after UV irradiating such infected cells during the period of viral DNA synthesis.

The report of Selsky, Weichselbaum and Little concerns the use of UV irradiated herpes virus to study cells from Bloom's syndrome patients, while Lytle and Goddard used protease inhibitors to find phenomenological differences between UV-enhanced reactivation of lambda phage in E. coli and that of herpes virus in CV-1 cells.

Andrew Rainbow presented adenovirus viral antigen data showing that damaged adenoviruses produce viral proteins more slowly upon infection of cells from people genetically predisposed to cancer than upon infection of cells prepared from apparently normal persons. Day and Ziolkowski showed fibroblasts from people having Cockayne's syndrome were only partly able to repair UV'd adenovirus 5 and that MNNG pretreatment of A498 cells decreased their ability to support the plaquing ability of MNNG treated adenovirus 5.

INDUCIBLE ERROR PRONE REPAIR IN MAMMALIAN CELLS?

The main tools used in this area were developed by C.D. Lytle and co-workers (1), and by Summers and Summers (2). Lytle and Goddard (unpublished, Fig.1) have recently found that a dose of UV to CV-1 AGMK cells enhances the

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mutability of infecting herpes virus whether or not the virus also receives UV irradiation. Unlike the situation with UV irradiated lambda phage infecting E. coli (3), UV irradiation does produce mutations in herpes virus even if the cells do not also receive radiation. Very similar results were obtained by Das Gupta and Summers (this volume and PNAS in press) using herpes virus type 1, strain C1-101 (from Saul Kit, Baylor) and Vero cells (ATCC), and Mouse LMTK cells (J. Eisenstadt, Yale) to use for the BrdUrd selection to TK minus herpes mutants.

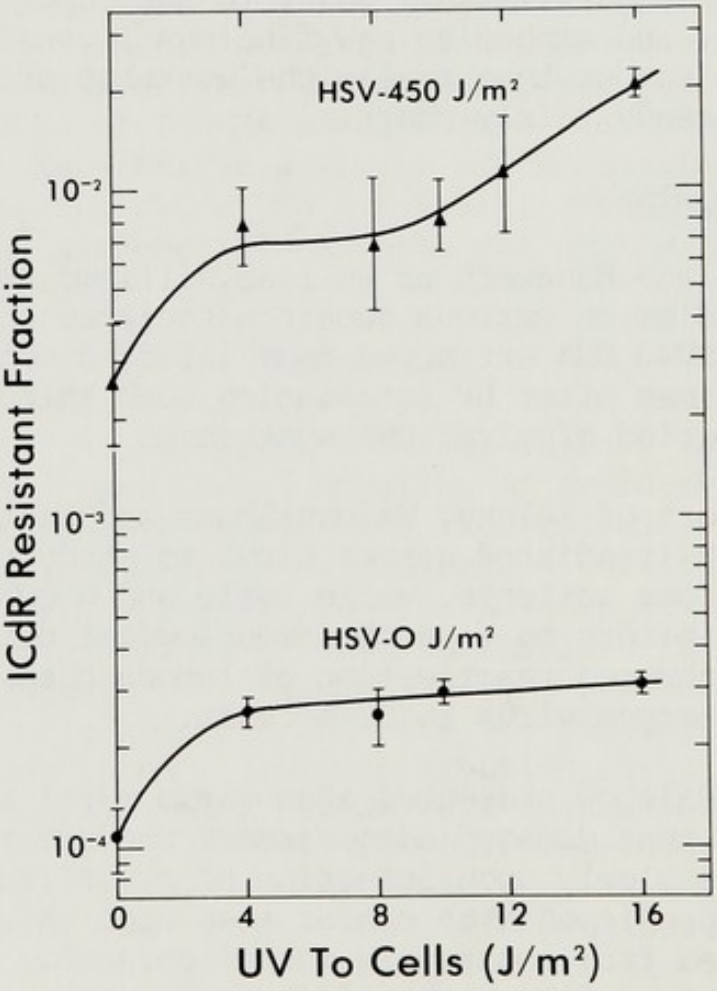

Fig.1. Mutation frequency (ICdR resistant fraction) of herpes virus as a function of UV dose to CV-1 cells 24 hours prior to infection. Herpes simplex, MP-1, was UV irradiated (germicidal), and used to infect (at low m.o.i. to minimize multiplicity reactivation) CV-1 AGMK (subclone TC-7) that had received the indicated UV doses 24 hours prior to infection. Viral yields were titered on CV-1 cells in the presence and absence of 40ug/ml ICdR. The CV-1 TK doesn't phosphorylate ICdR whereas the wild-type herpes TK does (2).

Together these reports, along with Lytle's extensive studies of UV enhanced reactivation of herpes virus (1) are strong evidence that inducible error prone repair occurs in two african green monkey kidney cell lines, CV-1 and Vero. However, the report of Day and Ziolkowski (this volume, showing little or no enhancement of the reversion frequency of adenovirus 5ts2 occuring when normal human fibroblasts are irradiated 24 hours prior to infection with the UV'd ts mutant, casts some doubt as to the generality of the phenomenon. As Paul Donovan (unpublished) points out, the possibility that UV-irradiation of cells results in the intracellular depletion of a cellular factor that (in unirradiated cells) reduces errors during herpes DNA synthesis must be excluded before inducible error-prone repair in mammalian is proved. Clearly there is room for work in this area. Until cellular mutants are made for the study of inducible error prone repair in mammalian cells (remember lex A is dominant) viruses remain powerful probe for asking experimental questions about the existence of such repair.

SURVIVAL OF DEPURINATED SV40 DNA

Kdruna, Linn, and Penhoet (unpublished results) have shown that the survival of depurinated (acid treated) SV40 DNA measured in an infectious centers assay using two xeroderma pigmentosum group D strains (ATCC, CRL 1200, CRL 1160) is actually greater than the survival of the same treated DNA using a normal human fibroblast strain. This unexpected finding appears to provide strong biological evidence for the earlier finding of Kuhnlein and Linn (4) that group D xeroderma fibroblasts are deficient in an apurinic site nuclease, although the biochemical basis for the increased survival of the treated DNA in the XP group D cells is not clear.

ENHANCED REACTIVATION

The phenomenon of increasing the surviving fraction of a damaged viral population by pretreating the cells (to be used for virus titration) with physical or chemical agents is called enhanced reactivation (ER). Three reports (5,6,7) have appeared which together support the idea that ER is a general phenomenon with respect to both the viruses and the physical chemical agents used. Thus ER of UV irradiated herpes (H), SV40 (S), and adenoviruses (A) has been observed when CV-1 african green monkey kidney cells

are pretreated with UV (H,S,A), x-rays (H,S,A), aflatoxin B1 (H,S), N-acetoxy-AAF (H,S), N-hydroxy-AAF (H), MMS (S) or EMS (S). It would be of interest to see results of similar work in which various cell sources and cell types were studied.

LYMPHOBLASTOID CELL LINES

Although not viral probes for repair, Earl Henderson and Jim German (this volume) have used Epstein-Barr virus to prepare lymphoblastoid cell lines using isolated blood lymphocytes taken from patients having Bloom's syndrome, ataxia telangiectasia, Cockayne's syndrome, and Fanconi's anemia. After partial characterization, the lines so far prepared appear to retain the relevant repair or chromosome breakage phenotypes characteristic of the non-transformed lymphocytes. Such lines, easily grown in quantity, should be extremely useful when biochemical characterization of repair defects becomes possible.

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STUDIES ON UV-INDUCED VIRAL REVERSION, COCKAYNE'S SYNDROME, AND MNNG DAMAGE USING ADENOVIRUS 5

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For the past several years our laboratory has been studying the repair by human cells of damaged human adenoviruses. For a review of our previous work in this area, see Ref. 1. Here we present an overview of three current projects. The first deals with the production of revertants by UV-irradiation of purified adenovirions; the second is a study of the repair defect in fibroblasts from patients having Cockayne's syndrome, and the third is a study of inactivation of adenovirus by N-methyl-N-nitro-N-nitroso-guanidine (MNNG).

Cleaver (2,3) has previously published data concerning the UV-induced reversion of an SV40 temperature sensitive mutant. We are beginning the study of the UV revertability of adenovirus using Ad5ts2, a temperature sensitive mutant of adenovirus 5 (4) and a human kidney carcinoma cell line, A498 (5). In an experiment designed in the manner shown in Fig. 1, using the A498 line as the cells under study, the results in Fig. 2 were obtained. The survival of the virus

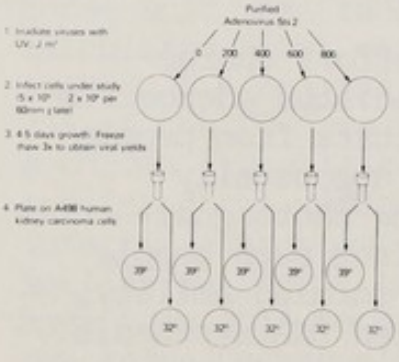

Fig. 1

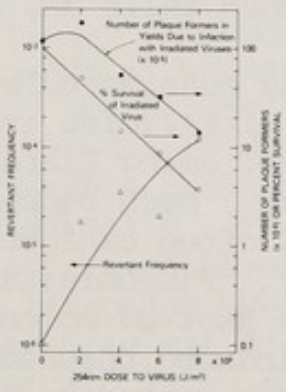

Fig. 2

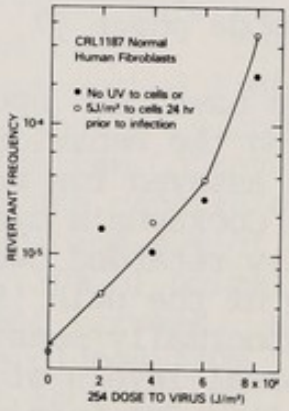

Fig. 3

in single infections of A498 cells is shown by the straight line and the open circles. Qualitatively, this curve resembles those obtained with normal human fibroblasts (6), showing that these cells can repair the UV-damaged viruses

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normally at 32 degrees. The UV-damaged viruses were also passaged through A498 cells at a multiplicity of about one in order to obtain viral yields, and these yields were titered and incubated either at 32 or 39 degrees. The plates incubated at 32 degrees gave the data shown by the closed circles. The rise in this curve has been noted in all such experiments so far completed, but its significance is not understood. The plates incubated at 39 degrees gave rise to revertant plaques, many of which grew to half-centimeter diameter, some to one centimeter, in the two-week plaquing period. However, we have not yet attempted to verify the temperature independence of viruses grown out from such revertant plaques. The number of such "revertants" was divided by the number of plaque forming units determined at 32 degrees to find the revertant frequency at each UV dose to the virus (open triangles, Fig. 2). In various experiments we have obtained qualitatively similar results of revertant production although quantitation has been difficult. Fig. 3 shows the results obtained with CRL1187 normal human fibroblasts as the cells under study. These were irradiated with 5 J/m (open circles) or not irradiated (black dots) one day prior to infection with irradiated virus. There were no revertants due to non-irradiated virus when the cells were not irradiated (as shown by the absence of a black point at zero dose). Because of the low numbers of revertants obtained at low UV doses in these experiments, we need to plate more viruses to obtain this point. But the current data suggest that irradiated cells do not give rise to a much greater revertant frequency than do non-irradiated ones, unlike the results obtained with either E. coli and lambda phage (7) or Vero cells and Herpes virus (8).

We have been spending considerable effort characterizing the behavior of ultraviolet irradiated adenovirus 5 when assayed for survival using fibroblasts from people having Cockayne's syndrome. Such people are usually mentally retarded dwarfs, often exhibiting photosensitivity of the skin (9,10). Fibroblasts from such people show abnormally sensitive post-UV colony forming ability, but normal levels of UV-induced unscheduled DNA synthesis (11, 12). Our initial work seemed to show a two-component inactivation of the virus as seen in Fig. 4. However, three of the cultures which were supplied to us were found to be contaminated with mycoplasma, which ultimately took over the lab. After recovering, we sought out more reliable cultures, obtaining one from Jay Robbins (GM1098,

grown from a biopsy taken from a patient, CS2BE, described in great detail in ref. 10) and one from Peter Nissley, both at NIH. Our result is shown in Fig. 5. It is clear

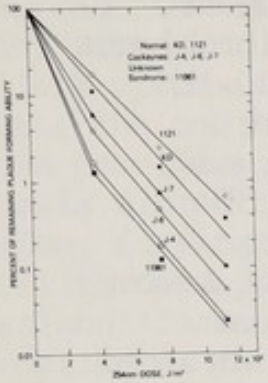

Fig. 4

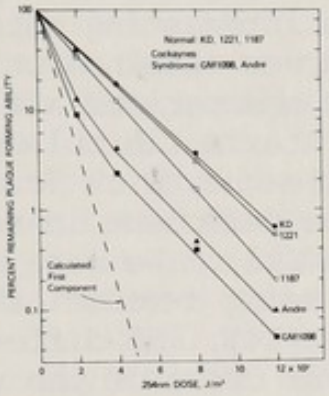

Fig. 5

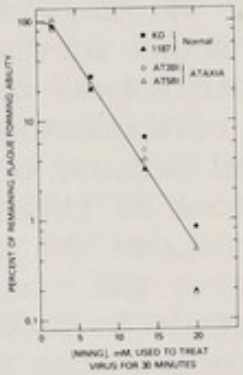

Fig. 6

that the inactivation of the virus using fibroblasts from people having Cockayne's syndrome does show two component behavior. The data plotted are average values obtained in two experiments for the normal strains, three for the Cockayne's. In all experiments the data obtained with the Cockayne's fibroblasts showed definite two-component behavior, while the normal strains showed only one component. The average one-hit inactivation dose using the normal fibroblasts is 218 J/m , and, in comparison, the more resistant component obtained using the Cockayne's fibroblasts has a value of 208 J/m , within the observed normal range. The more sensitive first component calculated from the data obtained using the Cockayne's fibroblasts has a one-hit inactivation dose of 63 J/m , so that the first component shows more than three-fold more sensitivity than does the second. The calculated first component is shown by the dashed line in Fig. 5. Analysis of the figure shows that only about 20% of the UV-damaged viruses which infect the Cockayne's syndrome fibroblasts, infect cells which are capable of repairing the viruses normally. The remaining 80% of the infected fibroblasts appear to have only 30% of normal ability to repair the infecting UV-damaged viruses. We have been able to grow three out of five clones from the Andre Cockayne's strain in an effort to test the possibility that these Cockayne's fibroblasts are heterogeneous with respect to their ability to repair UV-damaged adenovirus. So far, no viral inactivation data has been obtained with these clones.

We have made an effort to use MNNG inactivated adenovirus as a probe for finding human cells defective in their ability to repair alkylated DNA, but have been unsuccessful so far in finding such a repair deficient strain. We do have some interesting data, however. Fig. 6 shows survival curves obtained using fibroblasts from both normal persons and persons afflicted with ataxia telangiectasia. Ataxia fibroblasts were selected because our colleague, Nick Scudiero, has found some of these to be deficient in repair of MNNG produced DNA damage (13). We were able to inactivate the virus by using a modification of Lawley and Thatcher's procedure (14). No difference was found between the survival curve of the virus obtained using normal fibroblasts and that obtained using ataxia fibroblasts, showing that ataxia fibroblasts probably repair MNNG damaged viruses normally, although by Nick Scudiero's colony forming experiments (13), they are more sensitive than normal fibroblasts to MNNG inactivation. Similar data was obtained using the A498 cells (Fig. 7). Shoulders are usually, but not

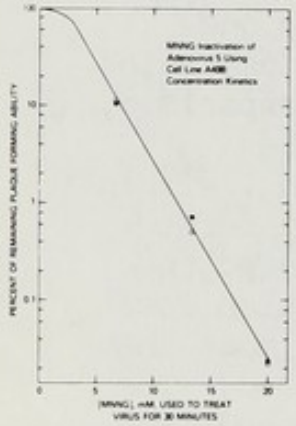

Fig. 7

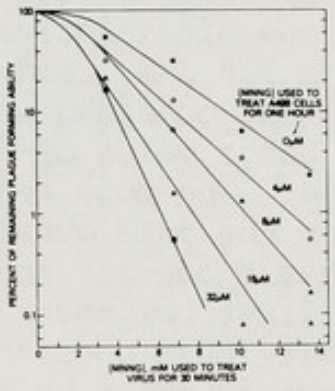

Fig. 8

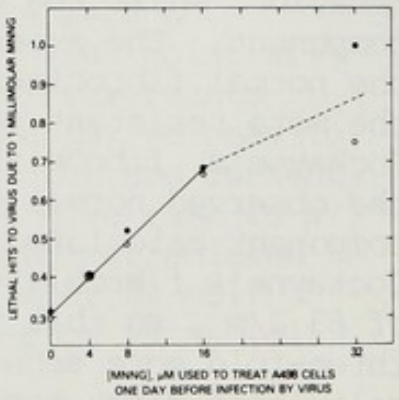

Fig. 9

always, obtained when the A498 cells were treated for one hour with the indicated concentrations of MNNG twenty-four hours before being infected with adenoviruses which had either received no treatment or which had been treated with various concentrations of MNNG. Such pretreatment of the cells specifically decreased their ability to support plaque formation by the MNNG treated viruses. The survival of UV-irradiated virus was actually increased somewhat by pretreatment with MNNG. We calculated the final slopes of these curves, and plotted these slopes as lethal hits due to 1mM MNNG versus the MNNG concentration used to pretreat the cells. (Fig. 9) In

one experiment, the effect saturated at about 16 uM MNNG. In the other it did not. 16uM is fairly toxic to normal human fibroblasts, giving about 10% survival of colony forming ability (13). The reason for the apparent decrease in repair seen here could clearly be due to many factors. We do know that the reduction is observed if the virus is used to infect cells within one hour after the one hour MNNG treatment. This observation does provide an explanation for the observation that ataxia fibroblasts have less than normal post-MNNG colony forming ability, but have apparently normal ability to repair MNNG treated adenovirus 5: The explanation would be that MNNG treatment of fibroblasts inactivates the repair system for MNNG produced DNA damage to a greater extent in ataxia than in normal fibroblasts. This, of course, is quite a testable hypothesis, which we have not yet checked.

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PRODUCTION OF VIRAL STRUCTURAL ANTIGENS BY IRRADIATED ADENOVIRUS AS AN ASSAY FOR DNA REPAIR IN HUMAN FIBROBLASTS[1]

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ABSTRACT A sensitive host cell reactivation (HCR) technique was used to examine the DNA repair ability of several different human fibroblast strains. Cell cultures infected with irradiated adenovirus were examined for the presence of viral structural (V) antigens using immunofluorescent staining. A reduced HCR of this viral function was detected following the infection of fibroblasts from several patients suffering from Fanconi's anaemia (FA), ataxia telangiectasia (AT), xeroderma pigmentosum (XP), and progeria (P) as compared to normal fibroblasts.

INTRODUCTION

HCR of irradiated adenovirus has been investigated using a variety of techniques including plaque formation (1,2,3,4,5), intranuclear inclusion body formation (6), repair of gamma ray induced single strand DNA breaks (7), and repair of UV-induced alkaline labile DNA lesions (8,9). It has been shown to express, in part at least, the repair mechanisms of the host cell and has been successfully applied in the detection of a defective repair mechanism for UV induced DNA damage in fibroblasts from patients with XP (3,4,5,6,9). XP is one of a number of autosomal recessive syndromes in man which is characterized by an increased chromosome instability and an increased incidence of cancer (10). These so-called "chromosome breakage" syndromes include FA, AT and Bloom's syndrome as well as XP. There are several lines of evidence which suggest some defect in DNA repair for all these syndromes. Another syndrome of considerable interest in regard to its DNA repair capacity is progeria (P), a rare human disease characterized by the appearance of premature and accelerated aging (11). We have examined the HCR of V antigen production for irradiated adenovirus in fibroblast strains from several of the above syndromes using immunofluorescent staining (12).

[1] Supported by the National Cancer Institute of Canada.

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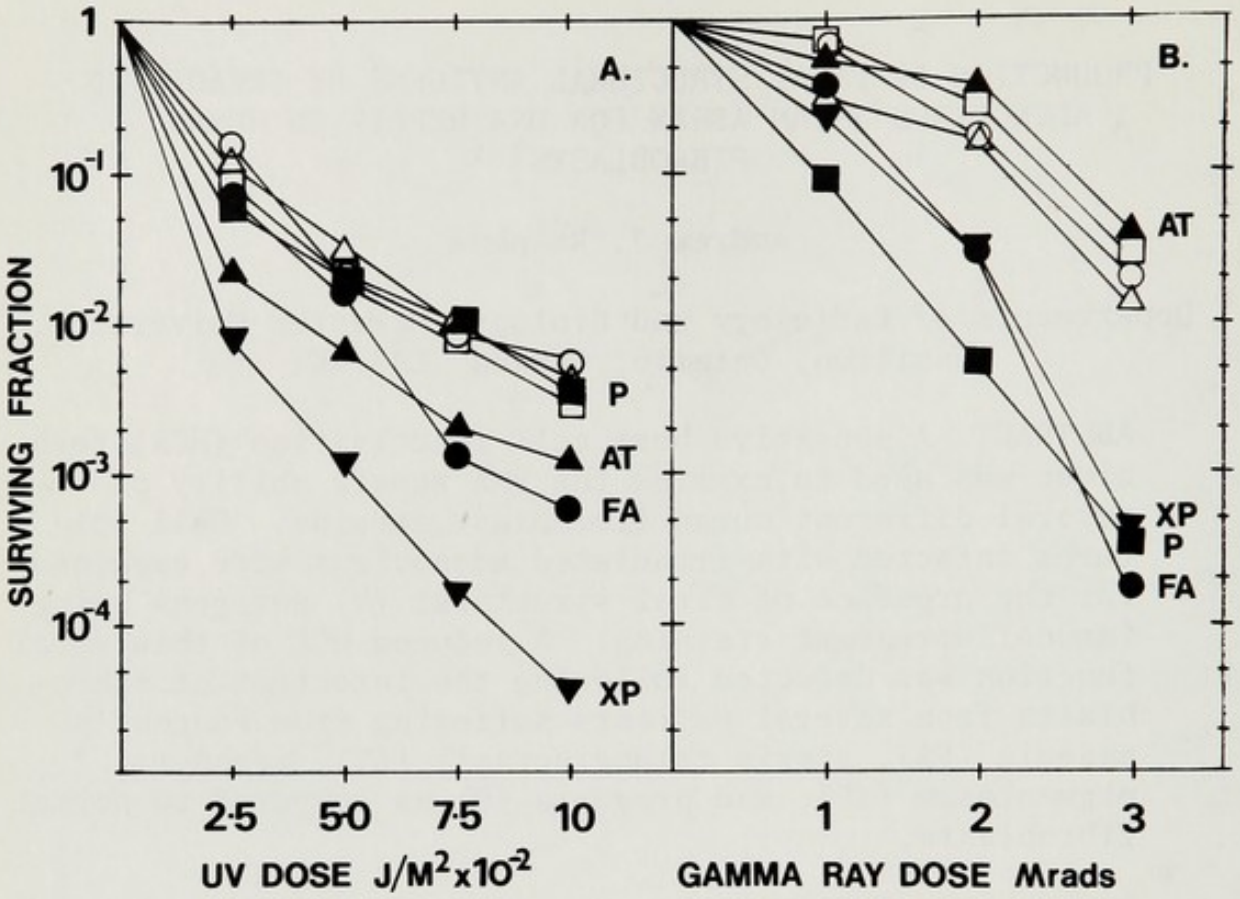

Figure 1. Host Cell Reactivation of Irradiated Adenovirus type 2 in Several Different Human Fibroblasts. A. UV-irradiation. B. Gamma irradiation. Normal: o CRL 1147; □ CRL 1119; Δ A2. FA: ● CRL 1196. P: ■ AK. AT: ▲ CRL 1347. XP: ▼ CRL 1204.

RESULTS AND DISCUSSION

Representative results for HCR of adenovirus type 2 (Ad 2) V antigen production at 48 hours after infection using 3 normal and 4 'patient' fibroblast strains are shown in Fig. 1. Since viral DNA synthesis is a requirement for the production of V antigens, the HCR examined in this way is a reflection of the DNA repair capacity required for DNA synthesis as well as the transcription and translation necessary for the synthesis of structural proteins (13).

A reduced HCR of UV irradiated Ad 2 has been reported by Day (3,4,5) for several XP strains using the endpoint of plaquing. The relative amount of host-cell reactivation for each of the XP complementation groups was found to correlate well with the relative amounts of UV-induced unscheduled DNA synthesis reported by Robbins & co-workers (14) with the exception of strains from group D which showed far less repair using the HCR assay. We have also detected a reduced HCR of UV-irradiated Ad 2 for the XP strains CRL 1204 (Fig. 1A) and

CRL 1160 (not shown) using the endpoint of V antigen synthesis. In agreement with Day's results we found that the HCR ability of the CRL 1160 strain (group D) was considerably less than that for CRL 1204 strain (group C). We have also detected a reduced HCR of V antigen production at 48 hours for gamma irradiated Ad 2 in several XP strains. The reduced HCR for gamma irradiation may be attributed to the known deficiences of XP for the repair of UV damage and suggests that some part of the DNA damage induced under these conditions of gamma irradiation mimics UV damage. XP cells have also been reported to be defective in repairing DNA damage induced by gamma irradiation under anoxic conditions (15).

Paterson & co-workers (16) have shown that some AT strains show a reduced amount of repair replication following gamma irradiation under anoxic conditions, whereas other AT strains, including CRL 1347, show apparently normal repair replication. This result has been used to suggest the existence of genetic heterogeneity in AT. As seen in Fig. 1, a reduced HCR for the AT strain CRL 1347 was detected following UV irradiation to the virus, whereas the HCR was not significantly different from normal for gamma irradiated virus. The former result was of considerable interest since AT strains have been reported to show normal colony survival following UV irradiation. Fibroblasts of this strain were also infected with either non-irradiated or UV irradiated Ad 12 and examined for the presence of V antigens at 48,72 and 96 hours post infection. Fig. 2A shows a significant reduction in HCR for the AT strain as compared to normal when assayed at either 48 or 72 hours post infection. However, the HCR of the AT strain was not significantly different from normal when assayed at 96 hours post infection. Fig. 2B shows a similar rate of V antigen production for unirradiated virus in both the AT and the normal strain. However, there is a considerable reduction in the rate of V antigen synthesis in the AT strain as compared to normal for UV-irradiated virus. These results suggest a reduced rate of repair for UV damaged DNA in this AT strain.

A deficiency in the repair of DNA damaged by either UV or gamma rays for the FA fibroblasts CRL 1196 has been reported previously (17). We have also detected a reduced HCR in 4 other FA strains (GM 1309, GM 391, GM 646 and GM 368) as compared to 2 normals (GM 409 and GM 726) when relatively large UV doses (10^3 J/M^2) were given to the virus (18).

We have also reported a reduced HCR of gamma irradiated Ad 2 for the progeria strains PG and AK (12). As shown in Fig. 1A, strain AK has apparently normal HCR following UV-irradiation of the virus. Little & co-workers (11) have reported a reduced capacity for the rejoining of gamma ray induced single strand DNA breaks in several progeria fibroblasts. The

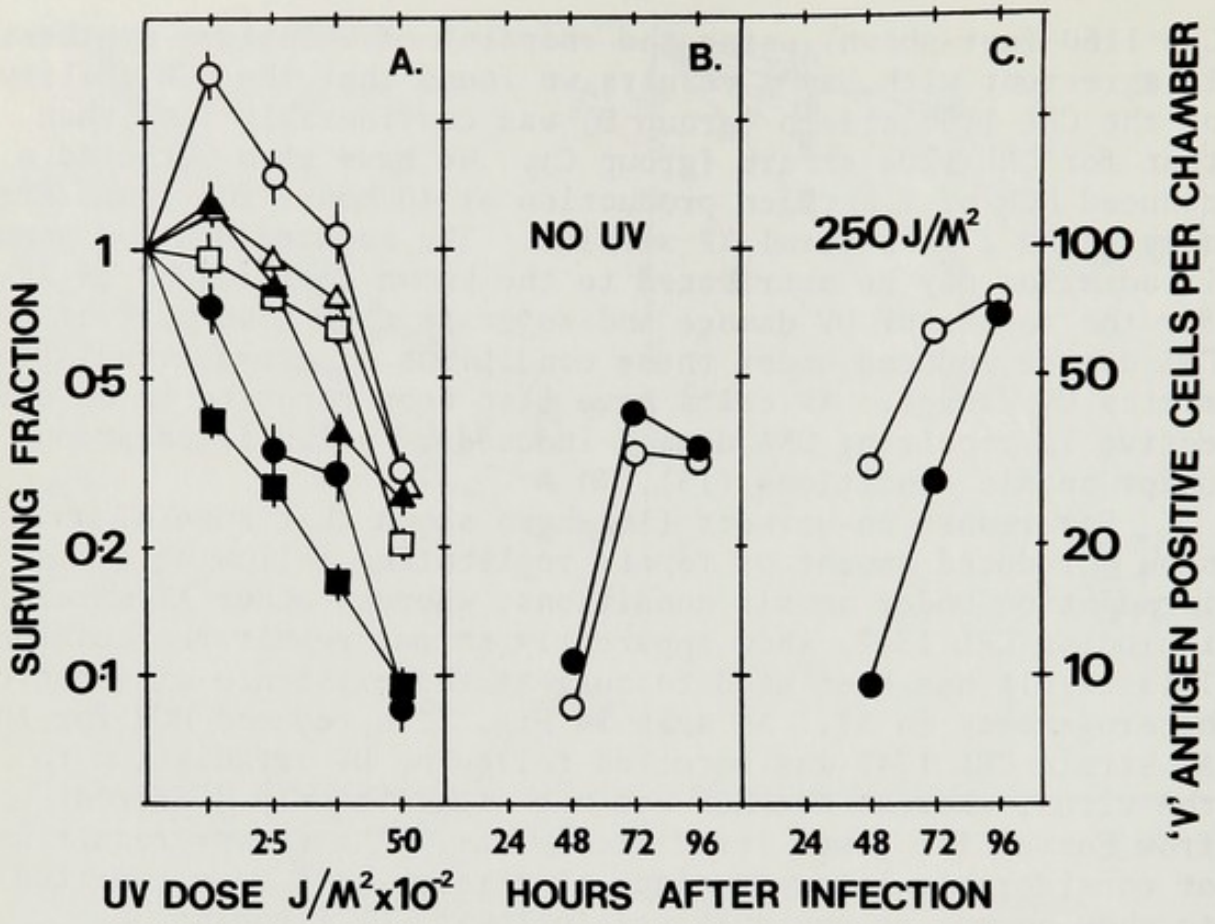

Figure 2. Host Cell Reactivation of UV-irradiated Adenovirus type 12 in Normal and AT Fibroblasts. Normal RE: open symbols. AT: CRL 1347 closed symbols. A: 48 hours o,●; 72 hours □,■; 96 hours Δ,▲.

reduced HCR for gamma irradiated Ad 2 may be a reflection of this reduced repair for single strand DNA breaks.

Comparison of Figs. 1A and 2A indicates a greater HCR for Ad 12 than for Ad 2 when assayed in the same cell line. This difference may reflect the longer time period between adsorption and viral DNA synthesis for Ad 12 (10-12 hours) as compared to 6-7 hours for Ad 2 (19). Repair mechanisms operating before the onset of DNA synthesis, such as excision repair would have a longer time period in which to remove DNA damage resulting in a greater HCR for Ad 12. The enhanced V antigen synthesis detected at 48 hours post infection following low UV doses to Ad 12 may be an expression of the same phenomenon which leads to enhanced tumor induction (20) and cell transformation (21) following irradiation of oncogenic viruses.

The results described here give a clear indication that the HCR of V antigen production for irradiated adenovirus is a sensitive assay for the DNA repair capacity of human fibroblasts capable of detecting repair deficiences where other methods have failed.

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SIMIAN VIRUS 40 AS A PROBE FOR STUDYING DNA REPAIR PATHWAYS IN MAMMALIAN CELLS[1]

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INTRODUCTION

Simian Virus 40 (SV40) can be used as a probe for studying constitutive and inducible DNA repair pathways in mammalian cells. This virus and its isolated DNA are easy to handle and its molecular biology is well documented. We have followed the fate of ultraviolet irradiated SV40 DNA in monkey kidney cells. This approach permits us to treat cultured cells by various chemical or physical agents without interfering with the DNA template to be repaired. We describe here the results obtained by employing this approach to examine (A) the effect of preconditioning the host cells upon survival of the damaged virus and (B) the mechanism of DNA replication on the damaged viral template.

(A) ENHANCED SURVIVAL OF UV-IRRADIATED SV40 IN CARCINOGEN-TREATED CELLS

We have shown that treatment of monkey kidney cells (CV-1P) with various chemical carcinogens enhances the survival of 1300 J/m^2-UV-irradiated SV40. The effective doses are very low and do not affect the capacity of cells to grow unirradiated viruses (1). UV-like compounds (N-acetoxy-acetylaminofluorene : AAAF, metabolites of aflatoxin B_1) and X ray-like compounds (methyl-methane sulfonate : MMS) are equally efficient and reactivation factors which quantify this enhancement vary roughly between 3 and 18 (Table 1).

Since UV-irradiation and carcinogen treatment are very efficient inhibitors of DNA synthesis and since some indirect inhibitors of cellular DNA synthesis, such as hydroxyurea or cycloheximide, also induce the enhancement of UV-irradiated SV40 survival in treated cells (1), we have hypothesized that DNA synthesis inhibition *per se* or a consequence of the DNA

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TABLE 1
REACTIVATION FACTOR OF 1300 J/m^2 UV-IRRADIATED SV40 AFTER TREATMENT OF CV-1P HOST CELLS BY VARIOUS CARCINOGENS

AAAF	Concentration	0	2	5	7.5 μM
	R.F.[a]	1	6.1	10.5	18.8
Aflatoxin B_1[b]	Concentration	0	8	16	24 μM
	R.F.	1	6.2	5.0	3.9
MMS	Concentration	0	0.5	1.0	2.0 mM
	R.F.	1	1.3	4.0	3.2

[a] Reactivation Factor = Ratio of the surviving fraction of UV-irradiated SV40 in treated cells to the surviving fraction of UV-irradiated SV40 in control cells.

[b] Aflatoxin B_1 has been metabolized by rat liver microsomal enzymes as previously described (4).

synthesis blockade is responsible for the enhanced survival of the viruses. This phenomenon might represent one of the expressions of "SOS repair" functions in eucaryotic cells analogous to the previously demonstrated induction of "SOS repair" in bacteria following UV or carcinogen treatment (2,3). It may occur by the action of an "induced" ability to bypass lesions in the viral DNA, thereby leading to higher survival. Is this process "error-prone"? That is the next question to be answered to evaluate the possible importance of this phenomenon in carcinogenesis. A preliminary report (5) suggests that mutagenesis of Herpes simplex virus is in fact associated with UV reactivation.

(B) DNA REPLICATION ON UV-IRRADIATED SV40 TEMPLATES

UV-irradiation (100 J/m^2) of SV40 DNA during a lytic infection in monkey cells (MA 134) inhibits viral replication. The synthesis of form I (supercoiled) SV40 DNA is inhibited more than 90% during the first hour after irradiation. Using the temperature sensitive SV40 mutant (tsA-58) to synchronize viral DNA replication prior to irradiation, we find that the first pyrimidine dimer is a block to DNA replication. The

presence of a pyrimidine dimer on one strand does not seem to inhibit DNA synthesis on the other strand however.

Pyrimidine dimers have been detected and quantified by using the endonuclease V activity from T4 bacteriophage which is specific for the DNA strand containing the dimers (6). Under our conditions, a 100 J/m^2 dose of UV-irradiation results in 7 pyrimidine dimers per SV40 genome. During the first hour after irradiation the size of the newly-synthesized DNA is equal to the distance between two dimers on the DNA template (about 0.50 x 10^6 daltons). Then, the size of the newly-synthesized DNA becomes larger and larger with time suggesting a bypass of the dimers (Table 2). There is no detectable exchange of dimers between "parental" and "daughter" DNAs and no detectable loss of pyrimidine dimers from parental DNA during the first 3 hours after UV-irradiation (Table 2).

The replicative intermediates isolated after UV-irradiation possess some unusual properties : a) the sedimentation in neutral sucrose gradients and the density value in CsCl-ethidium bromide equilibrium gradients show that the DNA template of these intermediates is relaxed or nicked rather than supercoiled as in "normal" replicative intermediates. b) These intermediates which contain pyrimidine dimers appear to be insensitive to the T4 endonuclease V.

TABLE 2
SIZE OF SV40 DNA DURING REPLICATION
AFTER 100 J/m^2 UV-IRRADIATION

Time after irradiation	Size of newly-synthesized DNA x 10^6		Size of "parental" DNA x 10^6 [b]
	No endo.	+ endo.[a]	+ endo.
0- 70 min.	0.47±0.03[c]	0.48±0.03	0.47±0.04
0-120 min.	0.56±0.05	0.59±0.05	0.48±0.03
0-220 min.	0.94±0.01	0.94±0.04	0.54±0.04

[a] The T4-endonuclease V treatment has been carried out as described (6).

[b] The size of single strand linear SV40 molecule has been taken as 1.8 x 10^6 daltons.

[c] Standard error on 2 to 4 determinations.

The mapping of the DNA synthesized after UV irradiation on the SV40 genome using the restriction endonuclease Hind III shows a gradient of radioactivity decreasing from the replication origin to the end of the genome. This result supports our model for SV40 DNA replication after UV-irradiation : from the origin of replication the DNA polymerase proceeds to the first dimer and stops. Then, after a long period (about 40-60 min. after the temperature shift), DNA synthesis resumes probably by-passing the dimer and going onto the next one. This replication could result in nicked, relaxed or supercoiled molecules. The bypass could be the result of the induced repair pathway described above.

CONCLUSION

SV40 can be used effectively as a probe to study DNA repair schemes in mammalian cells. Our results are consistent with the existence of an inducible repair mode that facilitates the eventual bypassing of pyrimidine dimers to permit the replication of the damaged DNA. This pathway can be induced either by a low dose of UV or by chemical carcinogen treatment of the host cells. It is likely that such a system would result in mutagenesis. Its possible role in carcinogenesis remains to be assessed.

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REPAIR OF UV IRRADIATION DAMAGE IN SIMIAN VIRUS 40[1]

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ABSTRACT The kinetics and extent of excision repair and DNA replication of UV-irradiated SV40 DNA were measured during lytic infection of monkey CV-1 cells. Loss of T4 endonuclease V-sensitive sites from viral DNA occurred in a complex, dose-dependent fashion and suggests damaged viral DNA was repaired by the host cell excision repair system. Pulse and pulse-"chase" labelling experiments revealed formation of SV40 Form I (supercoiled) DNA was the most sensitive step in viral DNA replication after UV irradiation. Label in replicative intermediate or Form II molecules "chased" effectively into Form I molecules even after 40 J/m^2 of UV light, indicating dimers ahead of the growing forks are bypassed without concomitant dimer removal.

INTRODUCTION

Viral probes of mammalian cell functions have provided valuable insights into the biochemical basis of DNA repair, mutagenesis and transformation. The papovavirus Simian Virus 40 (SV40) is especially well suited for deducing biochemical events because of its extremely small size (3.2 x 10^6 daltons), similarity *in vivo* to host cell chromatin in GC and histone content, and inability to code for any known DNA repair enzymes. Several recent studies have examined SV40 to establish a correlation between DNA repair capacity in mammalian cells and either host cell reactivation during permissive infection or induction of infectious virus from transformed cells (1-4). These studies, however, have used a biological endpoint (plaque formation) and shed little light on underlying biochemical events. Our studies used endonucleolytic cleavage and/or gel electrophoresis of SV40 DNA to study molecular events in excision repair and replication on UV-damaged SV40 templates.

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RESULTS

Comparison of UV-induced Excision Repair in SV40 DNA During the Lytic Cycle and in Host Monkey CV-1 Cell DNA. We previously reported the detection of pyrimidine dimers in Form I (supercoiled) SV40 DNA using dimer-specific T4 endonuclease V (5). Endonuclease-sensitive sites measured by agarose gel electrophoresis (6) were induced linearly at the rate of 0.049 dimers per SV40 genome per J/m^2 or 1.4 sites per 10^8 daltons of double-stranded DNA per J/m^2. Analysis of the dimer content in CV-1 DNA from uninfected cultures, indirectly with T4 endonuclease V and directly by thin layer chromatography, yielded values of 1.4 endonuclease-sensitive sites and 1.6 pyrimidine dimers per 10^8 daltons of DNA per J/m^2 (7). The similarity of these induction values supports our contention that endonuclease-sensitive site measurements accurately reflect the *in vivo* fate of pyrimidine dimers during post-UV incubation.

The dimer content in SV40 DNA decreased with time in a complex, dose-dependent fashion, but the kinetics at doses of 25-38 J/m^2 were generally biphasic with a relatively rapid phase over the first 4-8 hours and slower removal for the next 16-20 hours. The kinetics of endonuclease-sensitive site removal in CV-1 DNA were also biphasic over the same dose range (7). The number of endonuclease-sensitive sites removed from SV40 DNA in 24 hours was 10-14 sites per 10^8 daltons over the dose range 25-38 J/m^2, while 20-22 sites per 10^8 daltons were removed from CV-1 DNA in 24 hours over the same dose range (data not shown). The decreased extent of dimer removal in infected cultures may reflect the onset of cytopathic effects and cell death prior to cell lysis.

SV40 DNA Synthesis on UV-damaged Templates SV40 DNA was pulse-labelled with ^{3}H-thymidine (20 μCi/ml, 11 Ci/mmole) for twenty minutes after exposure of SV40-infected CV-1 cell cultures to 0-40 J/m^2 of UV light and either extracted immediately or after 1-3 hours of post-UV incubation in the presence of 10^{-5}M unlabelled thymidine and 10^{-5}M deoxycytidine. All native DNA conformations in the replicative cycle Form I DNA → replicative intermediate DNA → Form II DNA → Form I DNA were resolved in the extracted SV40 DNA by electrophoresis in 1.0% agarose gels (6). The presence of UV photoproducts inhibited tritium label uptake during viral replication by about 39% after 20 J/m^2 and 51% after 40 J/m^2. It can be shown that the predicted suppression of label uptake is about 21% after 20 J/m^2 and 47% after 40 J/m^2 if dimers ahead of the growing forks are absolute blocks to further nascent strand elongation during the tritium pulse. The general agreement in these numbers as well as the failure of label to accumulate in replicative intermediate or Form II SV40 molecules (Figure 1) indicates that DNA elongation

is the photosensitive step in the SV40 DNA replicative cycle. Post-UV incubation allowed label to accumulate in Form I molecules at a rate similar to that seen in unirradiated controls (Figure 1). The effectiveness of post-UV incubation in "chasing" tritium into Form I molecules suggests that many dimers are bypassed, presumably within one hour of the initial strand growth block since both replicative intermediates and Form II molecules decrease within this time. One alternative interpretation might be that molecules suffer absolute blocks of replication and the appearance of Form I molecules during post-UV incubation only reflects completion of replication in molecules with no dimers ahead of the growing forks. This is unlikely, however. The Poisson formula predicts the fraction F of all replicating SV40 molecules which carry at least one dimer in regions to be replicated is about $F=1-e^{-nf}$ where n is the mean dimer content per SV40 genome and f is the average unreplicated fraction of the genome. For a 20 min. pulse label $f \tilde{-} 0.13$ and consequently $F \tilde{-} 0.25$ at 20 J/m^2 and $F \tilde{-} 0.43$ at 40 J/m^2. These values seem too large to explain the proportions of Form I, Form II, and replicative intermediate molecules found after three hours of "chase" (Figure 1).

DISCUSSION

We have directly demonstrated that UV-induced DNA lesions

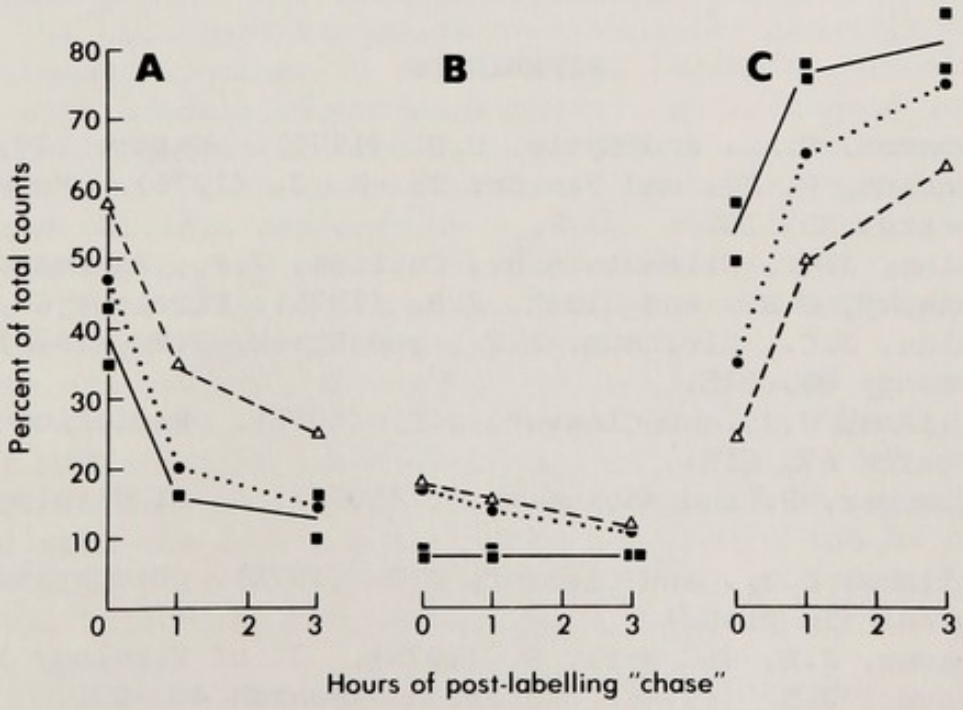

FIGURE 1. Percent total tritium counts in SV40 replicative intermediates (A), Form II (B) or Form I (C) DNA molecules after 0 J/m^2 (■——■), 20 J/m^2 (●---●), or 40 J/m^2 (Δ---Δ) and 0-3 hours of post incubation in unlabelled medium. Each data point is the mean value for three replicate cultures.

are removed from SV40 DNA by an excision repair pathway. This observation confirms the assumption of many host cell reactivation experiments, that viral DNA is as accessible to host cell DNA repair systems as host cell DNA itself. However, the presence of multiple copies of the SV40 genome in each cell may mean host cell reactivation is somehow related only to undamaged viral templates. It requires mutant host cell studies such as those with SV40 and xeroderma pigmentosum cells (1,2) to establish the importance of DNA repair for host cell reactivation.

Inhibition of SV40 DNA synthesis on UV-damaged templates is very much like "post-replication repair" in mammalian cells. Dimers are blocks to SV40 replication that are bypassed at a faster rate than dimers are released from SV40 DNA, suggesting replication across the dimer. Such replication could be error-prone and responsible for the increased rate of reversion to a wild-type phenotype in UV-irradiated SV40 that is correlated with the onset of replication during the lytic cycle (8,9). It is clear that SV40 will be useful in future biochemical studies of mutagenesis due to misrepair or lack of repair during replication in mammalian cells.

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DEFECTIVE HOST-CELL REACTIVATION OF UV-IRRADIATED HERPES SIMPLEX VIRUS BY BLOOM'S SYNDROME SKIN FIBROBLASTS[1]

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ABSTRACT Reactivation of UV-irradiated Herpes simplex virus was studied by infectious centers assay in three strains of skin fibroblasts derived from Bloom's syndrome patients. The results indicate that strain GM 1492 is defective in this process, but this defect does not correlate with increased cellular sensitivity to ultraviolet light. Caffeine had no apparent effect on either host-cell reactivation or clonogenic survival.

INTRODUCTION

The capacity of cells to support the growth of UV-irradiated herpes simplex virus (HSV) correlates with the cells' ability to repair UV-photoproducts in its own genome (1-3). This relationship has been demonstrated with xeroderma pigmentosum cells from the different genetic complementation groups with few exceptions (3) and has also been shown for the reactivation of UV-irradiated adenovirus (4).

Bloom's syndrome is an autosomal recessive genetic disorder with associated sun-sensitivity, an increased incidence of leukemia and an abnormally high degree of chromosome aberrations (5). It has been reported that cells from Bloom's syndrome patients demonstrate a slower than normal rate of chain growth during S-phase semiconservative DNA replication (6,7). The sun-sensitivity has not been correlated with deficient unscheduled DNA synthesis (8).

We are studying the reactivation ability of a number of Bloom's syndrome skin fibroblast cell strains for UV-irradiated HSV and have identified one strain to be defective. Clonogenic survival of this strain following UV-irradiation is normal over the dose range studied. Caffeine had no apparent effect on either UV-host-cell reactivation or clonogenic survival following UV-irradiation.

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METHODS

Bloom's syndrome skin fibroblast strains GM 2548, GM 2520 and GM 1492 were obtained from the Genetic Mutant Cell Repository, Camden, New Jersey. Normal skin fibroblast strains CRL 1116, CRL 1126 and xeroderma pigmentosum strains were purchased from the American Type Culture Collection, Rockville, Maryland. Normal skin fibroblast strain Li 106 has been maintained in this laboratory (9).

HSV-1 strain MP was obtained from the American Type Culture Collection. Growth and UV-irradiation of virus stocks have previously been described (3).

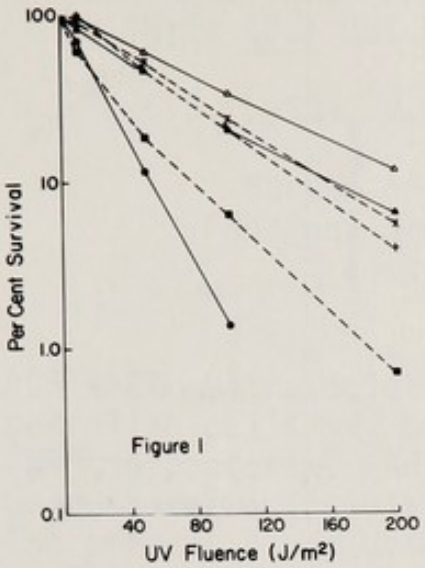
Survival of UV-irradiated virus in the various fibroblast strains was determined by assay of infectious centers. Fibroblasts were infected with unirradiated or UV-irradiated HSV at a multiplicity of infection = 3 based on the titer of unirradiated virus. Following a 90 minute adsorption period at 37°C., the inoculum was aspirated and the infected cells overlayed with Earle's balanced salt solution containing 0.16% pooled human immune serum globulin for 30 minutes at 37°C. Infected cells were then trypsinized and inoculated onto monolayers of HEp-2 cells in medium containing immune serum globulin. Incubation was for 72 hours at 37°C. The dishes were then stained with crystal violet and the plaques enumerated with the aid of a dissecting microscope. When the effect of caffeine on this system was studied, it was present in the medium for the entire 72 hour post-infection incubation.

The method for determining clonogenic survival of fibroblasts after ultraviolet-irradiation has previously been reported (9). Caffeine when used was present for the duration of the experiment following irradiation.

RESULTS

Figure 1 is a representation of the survival of UV-irradiated HSV-1 strain MP in Bloom's syndrome, normal and xeroderma pigmentosum cell strains determined by infectious centers assay. Inactivation kinetics of irradiated virus are similar for the two normal strains and Bloom's syndrome fibroblast strains GM 2548 and GM 2520. Strain GM 1492 was defective in some reactivation process as evidenced by a steeper inactivation curve for UV-irradiated virus in these cells. Caffeine at a concentration of 4mM in the post-infection assay medium had no effect on the kinetics of inactivation of irradiated virus in strain GM 1492 cells (Figure 2).

Figure 3 shows the clonogenic survival of GM 1492 and normal skin fibroblast strain Li 106. There is no evidence over the dose range studied that strain GM 1492 is abnormally sensitive to ultraviolet light. Caffeine present in the



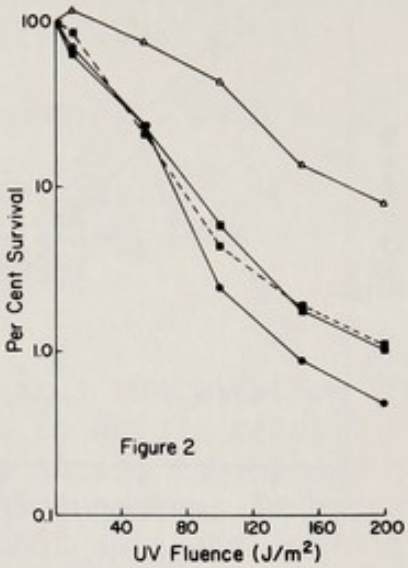


FIGURE 1. Survival of UV-irradiated HSV determined by infectious centers assay in △ - △ CRL 1116; ▲ - ▲ CRL 1126; ✖ - ✖ GM 2548; + - + GM 2520; ■ - ■ GM 1492 and ● - ● XP-C.

FIGURE 2. Survival of UV-irradiated HSV determined as in figure 1. △ - △ CRL 1116, ● - ● XP-C; ■---■ GM 1492 in the presence of 4mM caffeine, ■ - ■ GM 1492 without caffeine.

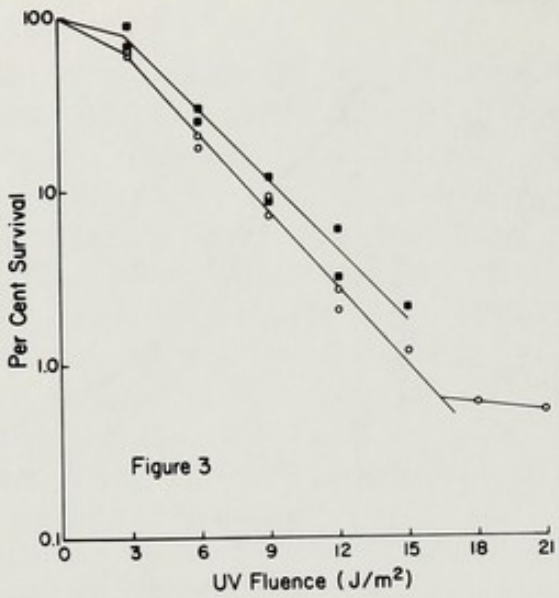
culture medium at a concentration of 2mM following ultraviolet-irradiation had no effect on the survival of these cells (Figure 4).

DISCUSSION

Skin fibroblast strain GM 1492 derived from a patient with Bloom's syndrome is defective in the reactivation of ultraviolet-irradiated Herpes simplex virus. Two other Bloom's syndrome skin fibroblast strains studied had normal reactivation ability. Clonal survival in these two strains could not be determined because of their relatively poor plating efficiency.

Strain GM 1492, defective in UV-host-cell reactivation, demonstrated normal clonogenic survival following UV-irradiation. Caffeine had no effect on either cell survival or host-cell reactivation. Studies are in progress to attempt the identification of some DNA repair defect that would correlate with the decreased host-cell reactivation exhibited by these cells.

Together, these results indicate the importance of



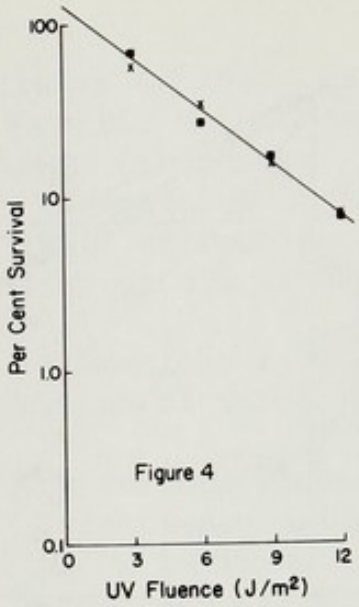


FIGURE 3. Clonogenic survival as a function of UV dose; ○ - ○ Li 106; ■ - ■ GM 1492.

FIGURE 4. Clonogenic survival of strain GM 1492 in the presence of 2mM caffeine ■ - ■ ; and in the absence of caffeine ✖ - ✖ , following UV-irradiation.

extensive phenotypic characterization of cell strains suspected of being defective in a DNA repair function.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Patricia Henson for her valuable comments and Mr. John Nove for his excellent technical assistance.

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PROTEASE INHIBITORS PREVENT UV-ENHANCED VIRUS REACTIVATION IN E. COLI BUT NOT IN MONKEY KIDNEY CELLS

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ABSTRACT Ultraviolet enhanced virus reactivation (ER) in mammalian cells may be analogous to Weigle reactivation (WR) in bacteria. Antipain, a protease inhibitor, has been shown to inhibit several SOS functions in bacteria. The present study demonstrates that antipain inhibits WR of UV-irradiated λ phage in E. coli C600. A similar experiment performed with CV-1 monkey kidney cells and herpes simplex virus determined that ER of UV-irradiated herpesvirus was not inhibited by protease inhibitors, antipain or leupeptin. Thus, the similarities between WR in E. coli (10) and ER in monkey kidney cells do not extend to inhibition by antipain. Furthermore, if ER is a mammalian cell SOS function and is regulated by a protease, that protease is not inhibited by antipain or leupeptin in CV-1 cells.

INTRODUCTION

Among the candidates for mammalian cell processes which may qualify as SOS functions is enhanced virus reactivation (see references 1 and 2 for review). Pretreatment of CV-1 monkey kidney cells (1) with UV or x radiations, photodynamic action or carcinogenic chemicals (3) results in enhanced survival of UV-irradiated herpes simplex virus. Enhanced virus reactivation (ER) in mammalian cells may be analogous to Weigle reactivation (WR), an SOS function in bacteria.

The SOS functions in E. coli have common regulatory pathway(s) (2). It has been postulated that proteases play a role in this regulation by irreversible inactivation of repressors of the SOS functions. Evidence for the role of proteases was provided when Meyn et al. (4) demonstrated that antipain, a protease inhibitor, blocked several SOS functions in E. coli.

The purpose of the present study was to test whether antipain could inhibit WR in E. coli or ER in monkey kidneycells. The results demonstrate that antipain inhibits WR in E. coli, but not ER in monkey kidney cells.

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METHODS

Materials. We used two protease inhibitors, antipain and leupeptin (5). Stock solutions at 100 mM were prepared in distilled water by filter sterilization.

The bacterial experiments were performed with E. coli C600, E. coli AB1886, and λ phage (wt). All cell growth was in TBMM medium (1% tryptone broth supplemented with 0.5% NaCl, 0.2% maltose, 10 mM $MgCl_2$, and approximately 1 μg/ml vitamin B_1). For λ phage plaque assay, the standard double layer plating technique was used, employing 1.5% and 0.75% agar for the bottom and top layers, respectively, in the TBMM medium without maltose. Bacteria or phage were irradiated in phosphate-buffered saline with 10 mM $MgCl_2$. E. coli AB1886 was used as lawn bacterium.

The mammalian cell experiments were performed with CV-1 (TC7) African green monkey kidney cells and herpes simplex virus (MP strain). Cell growth, virus growth and assay procedures have been published (6).

Procedures for irradiation with a germicidal lamp (254 nm) and dosimetry have been published (6).

Experimental Procedure. Experimental demonstration of ER or WR consists of incubating UV-irradiated cells to allow maximal expression of the enhanced reactivation ability and then assaying the reactivation ability by infecting these cells with UV-irradiated virus and determining the virus survival by plaque formation (1).

RESULTS

Inhibition of WR in E. coli. Weigle reactivation of UV-irradiated λ phage was easily demonstrated in E. coli C600. Incubation of UV-irradiated cells for 30 minutes before phage infection gave maximal WR and was used in this study. The antipain was present during this 30 min. incubation, during the 60 min. phage adsorption period at 4° C, and in the top agar during the plaque assay. The presence of antipain in the bottom agar had no effect on the results.

The effect of different concentrations of antipain on plaque formation by UV-irradiated and unirradiated phage after infecting UV-irradiated or unirradiated bacteria is shown in figure 1. Antipain caused a small decrease in the number of plaques caused by unirradiated phage in irradiated or unirradiated bacteria.

The results with irradiated phage were considerably different. In unirradiated bacteria, the survival of the irradiated phage increased with low concentrations of antipain. The cause of this increase is unknown.

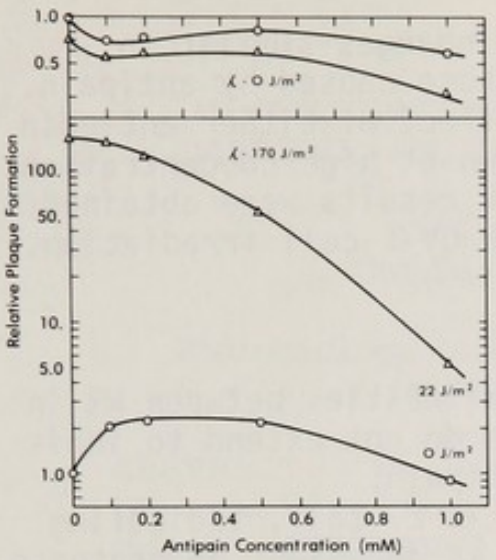

Figure 1. Effect of antipain on Weigle reactivation of UV-irradiated (170 J/m^2) λ phage in UV-irradiated (22 J/m^2) E. coli C600: relative plaque formation in irradiated (Δ) and unirradiated (o) bacteria. The surviving fraction of the irradiated phage was 4 x 10^{-5} in unirradiated cells without antipain.

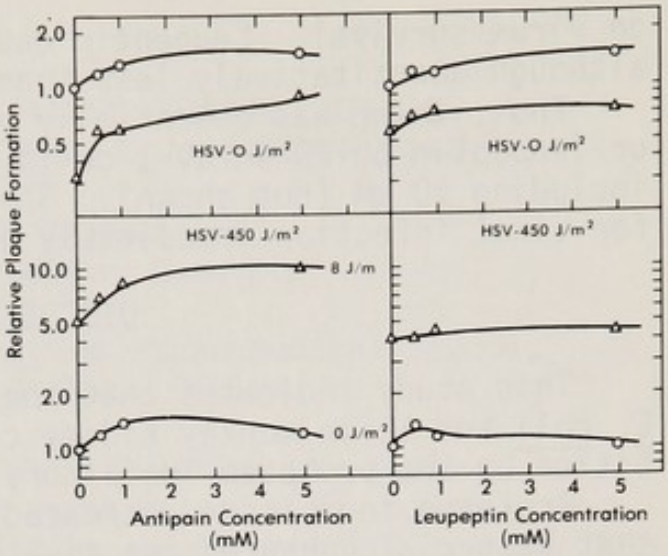

Figure 2. Effects of antipain and leupeptin on enhanced reactivation of UV-irradiated (450 J/m^2) herpes simplex virus in CV-1 monkey kidney cells. Cell doses: o, 0 J/m^2; Δ, 8 J/m^2. Surviving fraction of irradiated virus was 10^{-3} in unirradiated cells without chemicals.

Weigle reactivation is clearly evident for irradiated phage in irradiated bacteria, with an increase of survival by more than two orders of magnitude. Antipain at 0.5 and 1 mM caused a decrease in the UV-increased survival, indicating an inhibition of WR.

Lack of Inhibition of Enhanced Virus Reactivation in Monkey Kidney Cells. Enhanced survival of UV-irradiated herpes simplex virus in UV-irradiated CV-1 cells is maximal for viral infection 1-2 days after cell irradiation (3,7); we used 24 hours, which resulted in the increased virus survival shown in figure 2. The protease inhibitors, antipain or leupeptin, were present in the culture medium during the 24 hour post-irradiation incubation period and the subsequent viral infection. The inhibitor concentrations used were equal to or greater than those used with the bacteria.

Antipain caused smaller plaque size (not shown) and small increases in number of plaques by irradiated and unirradiated virus, particularly in irradiated cells. However, the increases were approximately equal for irradiated and unirradiated viruses, yielding little, if any, effect of the antipain

on virus survival. Leupeptin caused changes similar to, although quantitatively less than, those caused by antipain.

Thus, there was essentially no effect of either antipain or leupeptin on ER in CV-1 cells, even at high concentrations, including 10 mM (not shown). Similar results were obtained for viral infection immediately after CV-1 cell irradiation.

DISCUSSION

This study indicates that the similarities between WR in E. coli and ER in monkey kidney cells do not extend to inhibition by the protease inhibitor, antipain.

Protease inhibition decreased WR in E. coli, indicating that bacterial enhanced reactivation is dependent on protease action. Thus, this study provides further evidence that proteases play a role in bacterial SOS functions.

However, neither antipain nor leupeptin had appreciable effect on ER of HSV in monkey kidney cells. While this result suggests that proteases may not play a role in ER in mammalian cells, it must be noted that protease inhibitors can be quite specific for different proteases (e.g., ref. 8). Thus, if a protease is involved in ER in CV-1 monkey kidney cells, it is not inhibited by antipain or leupeptin.

ACKNOWLEDGMENTS

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UV-REACTIVATION OF HERPES SIMPLEX VIRUS IS MUTAGENIC AND INDUCIBLE IN MAMMALIAN CELLS [1]

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ABSTRACT: We have studied the processes of UV-reactivation and mutagenesis in mammalian cells with HSV-1 as the experimental probe. The survival of plaque-forming ability was measured along with the frequency of forward mutation to the TK$^-$ (thymidine kinase deficient) viral phenotype. These studies suggest that that UVR and mutagenesis are in some way inducible, and may be tightly coupled phenomena.

INTRODUCTION

In some procaryotes, agents such as UV, which damage DNA, result in induction of prophage, reactivation of irradiated virus grown on such cells, and mutagenesis of the cell as well as viruses grown in these cells, e.g., review by Witkin (1). We are interested in the existence of such DNA damage-activated phenomena in mammalian cells and have tested the idea that prior UV irradiation of the host cells results in a mutagenic state. To do this, we have carried out experiments in an animal virus system (herpes simplex virus, type 1) which are analogous to those of Weigle (2) with bacteriophage lambda. A more detailed account of these experiments will be published elsewhere (3).

RESULTS

We have measured the frequency of mutations in the thymidine kinase gene (TK) (for methods see refs. 3,4) of herpes simplex virus (HSV) following one cycle of growth on normal and UV-irradiated mammalian cells (Vero, an African green monkey kidney cell line). Prior irradiation of the host cell results in an increase in mutant frequency in the progeny virus. This result is obtained for both irradiated virus as well as unirradiated virus (Figure 1). In order to observe

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this mutagenic effect, the irradiation of the host cells must be done several hours prior to infection with the virus; 6 to 16 hours were necessary to achieve the maximum effect in various experiments

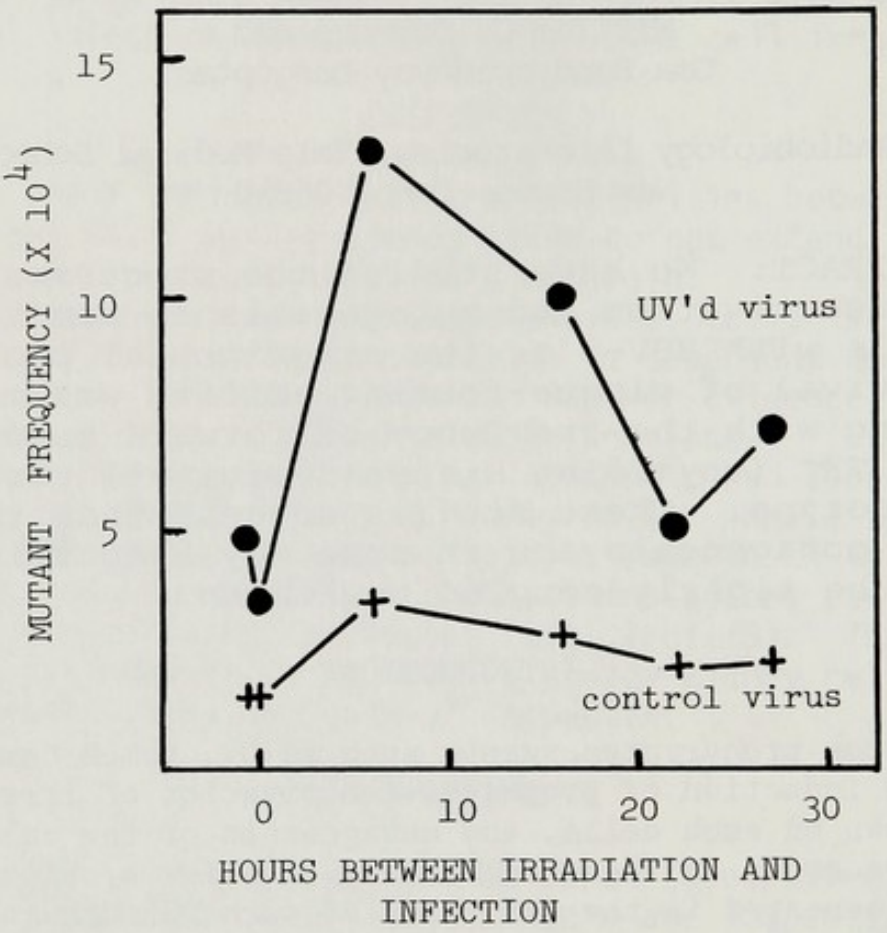

FIGURE 1. Frequency of TK^- mutant virus in progeny stocks following growth on Vero cell monolayers that had been irradiated with 103 ergs/$mm^2$from a GE germicidal lamp. The first point at -0.5 hours represents the frequency obtained with virus grown on control, unirradiated cells. The time interval between UV-irradiation of the cells and virus infection was varied. Both unirradiated virus and UV-irradiated (1840 ergs/mm^2) HSV were used as indicated.

Measurements of survival of UV-irradiated HSV made in parallel to the mutagenesis experiment allowed calculation of the UV-reactivation factor. The kinetics of appearance of the post-irradiation mutagenic state was parallel to that for UV-reactivation.

To test the hypothesis that the development of the mutagenic state requires the synthesis of new protein after UV irradiation, we added the protein synthesis inhibitor cycloheximide (10ug/ml) to the cell cultures at various times.

If we carried out infections at 8 hours after UV irradiation of the host cells, the UV-reactivation factor was 2.0; similarly, the increase in mutant frequency was two or three times that found for virus grown immediately after irradiation of the host. When cycloheximide was present between irradiation and infection, however, both the reactivation and the mutation frequency increase was not observed.

These results suggest that UV-irradiation of the host cells (Vero) can "induce" states of enhanced repair of irradiated HSV, and increased mutagenesis of both irradiated and unirradiated HSV. This induction phenomenon can be prevented by the protein synthesis inhibitor, cycloheximide.

DISCUSSION

Although there are obvious parallels between the results of our experiments and those obtained in bacteria, we still do not know the mechanism responsible for mutagenesis and reactivation of viruses grown on UV-irradiated mammalian cells. The inhibition of the mutagenic state by inhibition of protein synthesis is not conclusive evidence that UV irradiation induces the synthesis of a new protein(s) which mediate the reactivation and mutagenesis. Alternative explanations are possible. Prolonged exposure to cycloheximide after UV irradiation certainly puts the cell in a grossly abnormal physiologic state. Non-specific inhibition of the repair pathways could easily account for the observed results.

The kinetics of appearance of the mutagenic state, however, argues that some rather slow physiologic process must occur after irradiation to allow the cell to reactivate the damaged virus and to allow replication of the virus with less than the normal fidelity.

The results of our experiments suggest that the model of "error-prone" repair and replication of DNA in UV irradiated cells may hold for mammalian cells as well as bacteria.

ACKNOWLEDGEMENTS

We thank Dr. Wilma P. Summers for much help and advice with HSV TK genetics and cell culture methods. L. Wilkinson, L. Kalghatgi, and N. Logan all provided invaluable technical assistance. This work was initially stimulated by a seminar presentation at Yale in Feb. 1977 by Dr. R.B. Setlow.

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THE EFFECT OF DNA DAMAGE ON THE INDUCTION OF SIMIAN VIRUS 40 (SV40) IN TRANSFORMED HAMSTER CELLS[1]

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ABSTRACT The relationship between DNA repair processes and the induction of infectious virus in SV40-transformed hamster kidney cells has been investigated. Caffeine was found to inhibit postreplication repair in SV40-transformed hamster cells in which caffeine enhanced the induction of virus synthesis by UV light or mitomycin C. It is suggested that the persistence of gaps in DNA, caused by the presence of caffeine, results in the enhancement of SV40 virus induction.

INTRODUCTION

We have described SV40-transformed hamster kidney cells (clone A and clone E) in which treatment with mitomycin C, bromodeoxyuridine and visible light, UV light or ionizing radiation, results in the induction of infectious virus (1). These agents, which produce breaks or gaps in DNA strands either directly or indirectly via DNA repair mechanisms, induced virus in amounts proportional to the dose used. Recent experiments have demonstrated that exposure to caffeine significantly increases the level of virus induction by UV light (2). Caffeine inhibits the filling of gaps in newly synthesized DNA during postreplication repair and causes the accumulation of low molecular weight DNA in certain mammalian cell lines (3,4). Having hypothesized that DNA strand breakage is an important early step in virus induction, we investigated the effect of caffeine on postreplication repair of UV light damaged DNA in the SV40-transformed cell lines in which caffeine enhanced virus induction. Since it has been proposed that alkylated DNA bases may be repaired by a caffeine-sensitive postreplication repair mechanism (5), the effect of

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caffeine on virus production in cells treated with mitomycin C has also been studied.

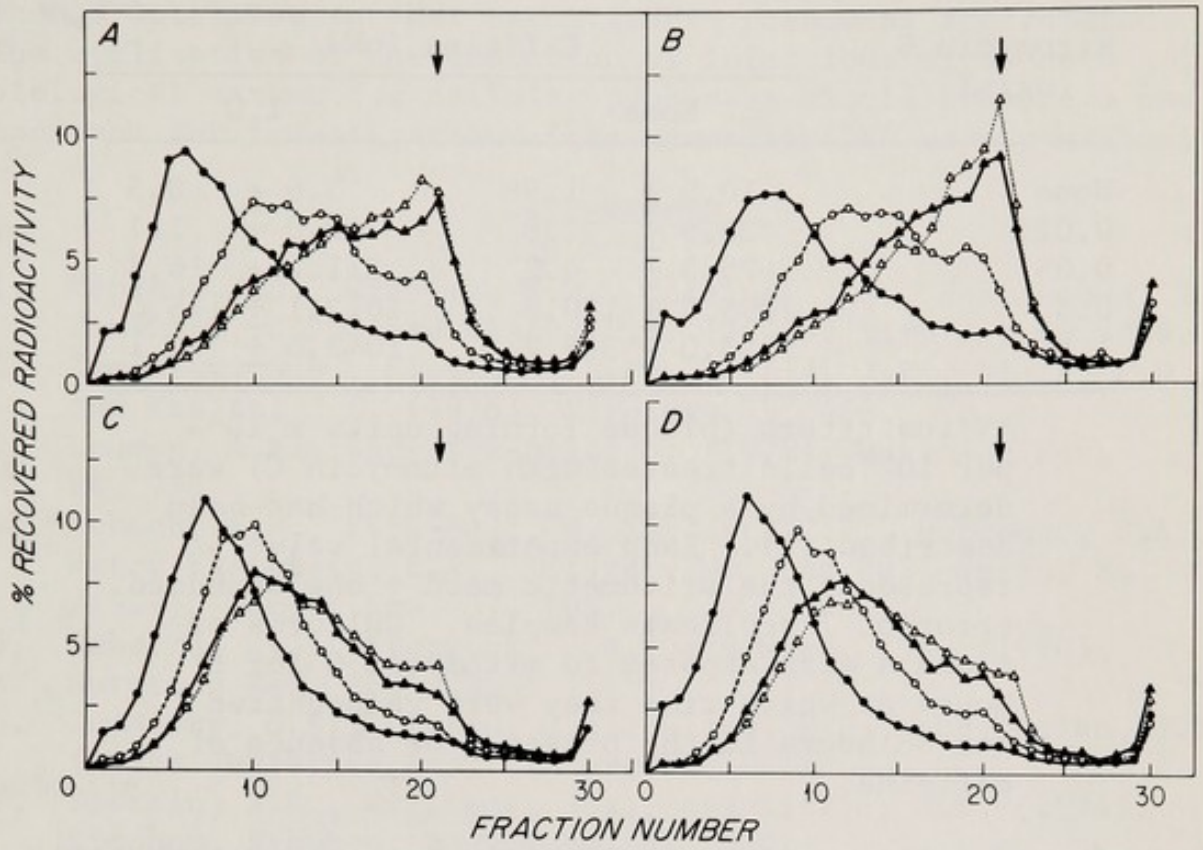
RESULTS

Effect of Caffeine on Postreplication Repair in Clones A and E. Prior results had indicated that treatment of UV-irradiated cells with caffeine increased virus induction in clones A and E as much as 10-20 fold (2). In order to investigate the mechanism responsible for this enhancement, the effect of caffeine on postreplication repair of UV light damaged DNA was studied by use of alkaline sucrose gradient sedimentation. Figures 1A-1D illustrate gradient profiles of newly synthesized DNA from UV-irradiated cells of clones A and E. As Figures 1A and 1B demonstrate, a large percentage of pulse-labeled DNA in UV-irradiated cells was chased into parental size DNA molecules by 5-8 hours. Exposure to 1 mM caffeine immediately following UV irradiation inhibited post-replication repair, preventing DNA molecules from reaching parental size (Figures 1C and 1D). This inhibition of post-replication repair was observed at chase times as short as 2 hours as demonstrated by the sedimentation of DNA at higher positions in these gradients than in those of cells which received no caffeine. Caffeine did not inhibit daughter strand growth in non-irradiated cells of clones A and E (data not shown).

Effect of Caffeine on Virus Induction by Mitomycin C in Clone A. It has been suggested that alkylated DNA bases are repaired by a caffeine-sensitive postreplication repair mechanism similar to that which repairs UV light damaged DNA (5). If the mechanism responsible for the inhibition of repair is the same in cells treated with UV light or alkylating agents, exposure of mitomycin C treated cells to caffeine should enhance SV40 virus induction. This was investigated in clone A cells. As seen in Table 1, exposure to 1 mM caffeine increased the level of virus production 2-3 fold in clone A cells grown for 8 hours in medium containing 0.025 ug/ml or 0.05 ug/ml mitomycin C. The decreased yields of virus observed at 0.5 ug/ml and 1.0 ug/ml mitomycin C were probably due to the extreme degree of toxicity of treatment with caffeine at these higher mitomycin C concentrations. Caffeine (1 mM) substantially decreases survival of mitomycin C treated clone A cells (6).

DISCUSSION

We have demonstrated that caffeine inhibits postreplication repair in SV40-transformed cell lines in which caffeine enhances virus induction by UV light or mitomycin C. Treatment with caffeine alone did not stimulate virus induction in



Figures 1A-1D. Alkaline sucrose gradient profiles of newly synthesized DNA from UV-irradiated clone A and clone E cells. Clone A and clone E cells were grown on plastic coverslips and lysed directly on 5-20% linear alkaline sucrose gradients by the technique of Epstein et al (7) as modified by Zamansky et al (6). Cells, prelabeled for 24 hours with 1 uCi/ml ^{14}C-deoxythymidine (specific activity, 40-60 mCi/mmol), were irradiated with 10.0 J/m^2 of UV light. The irradiated cultures were incubated in the presence or absence of 1.0 mM caffeine, pulse-labeled for 60 minutes with 30 uCi/ml ^{3}H-deoxythymidine (specific activity, 40-60 Ci/mmol), chased for 0, 2, 5, or 8 hours, and lysed. Lysis was followed by centrifugation at 5000 rpm for 15 minutes and 36,000 rpm for 75 minutes. The direction of sedimentation was from left to right. (A) clone A, no caffeine; (B) clone E, no caffeine; (C) clone A, 1.0 mM caffeine; (D) clone E, 1.0 mM caffeine. Chase times: 0 hr ● --- ●; 2 hr 0 --- 0; 5 hr ▲ --- ▲ ; 8 hr △ --- △ . Arrows indicate the peak fraction of ^{14}C prelabeled parental DNA.

TABLE 1
EFFECT OF CAFFEINE ON MITOMYCIN C INDUCTION OF SV40 VIRUS IN CLONE A

Mitomycin C (ug/ml)	Caffeine (mM)	
	None	1.0
None	10.9 ± 1.9*	5.6 ± 0.5
0.025	20.9 ± 2.8	40.0 ± 1.7
0.05	75.3 ± 4.2	211.2 ± 16.3
0.5	2065.0 ± 120.6	1612.1 ± 145.0
1.0	5553.0 ± 368.2	1042.0 ± 62.1

*Virus titers (plaque forming units x 10^{-2} per 10^6 cells treated with mitomycin C) were determined by a plaque assay which has been described (1). Each experimental value represents the arithmetic mean ± one standard error of 7 replicate samples. Cultures of clone A were exposed to mitomycin C for 8 hours at which time they were reincubated for 88 hours in the presence or absence of caffeine.

transformed cells or virus replication in a permissive cell line (2). We believe that it is the persistence of gaps in cellular DNA which results in the caffeine enhancement of SV40 virus induction.

The means by which discontinuities in DNA molecules result in the induction of SV40 remains unclear. It has been suggested that excision of the SV40 genome from host cell DNA occurs in cells induced by mitomycin C or ionizing radiation (8). Gaps in DNA strands may lead to conformational changes in DNA molecules such that the excision of the viral genome occurs more readily. Sussman and Ben Zeev have proposed another role for breaks in DNA. In their model of bacteriophage lambda induction in *E. coli*, it is suggested that breaks in DNA strands lead to dilution of the lambda repressor by its binding to nicked DNA, thereby inducing the phage (9). Such a mechanism may also prove to be important in mammalian cells.

The presence of DNA strand discontinuities may be indirectly responsible for the induction, activation or derepression of host enzymes capable of replicating the integrated viral genome in SV40-transformed cells. It is possible that breaks or gaps in mammalian DNA induce an "SOS" type repair system similar to that described for prokaryotes in which agents responsible for inducing "SOS" repair also induce lysogenic phage. Recent evidence suggests that treatment with UV light

or X-radiation induces DNA repair functions in mammalian cells (10,11,12). We have demonstrated that these agents induce SV40 in clones A and E. Further investigations into the relationship of DNA repair and the induction of SV40 virus should determine to what extent these phenomena are related. The utilization of the induction of infectious virus as a biological marker for cellular processes should provide a new approach for investigations into mammalian DNA repair mechanisms.

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RECENT DEVELOPMENTS IN THE BIOCHEMISTRY OF GENETIC RECOMBINATION[1]

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ABSTRACT An *in vitro* system is described in which the cutting of crosslinked ØX RF I DNA molecules by the *uvr* system of *E. coli* induces the cutting of homologous undamaged DNA during incubation with crude extracts of thermally induced *E. coli* (λ *precA*$^+$) lysogens. This reaction, which has also been observed in intact *E. coli* lysogens infected with λ phages, is dependent on the presence of functional *recA*$^+$ and *uvrB*$^+$ gene products.

INTRODUCTION

According to recent models (1), genetic recombination is initiated by invasion of a double-stranded DNA molecule by a single strand displaced from a homologous DNA molecule, followed by enzyme cleavage of the triple-stranded complex thus formed.

This hypothesis has received considerable support from the work of P. Ross and P. Howard-Flanders (2, 3 and this volume). Using E. *coli* (λ) lysogens superinfected with a mixture of intact and crosslinked λ phages, they showed that the cleavage of the damaged phage DNA by *uvr* system of *E. coli* induced the cutting of the undamaged phage DNA. This reaction, which they called, 'cutting in *trans*', was shown to be dependent on functional *uvr* and *recA* genes and to occur only between homologous DNA molecules.

Since genetic recombination in *E. coli* is totally dependent on the *recA* gene, and since crosslinks are known to produce abundant genetic exchanges in phage-prophage crosses in wild type lysogens (4), 'cutting in *trans*' is likely to represent an early step in recombination.

In order to investigate the mechanism of genetic recombination and to identifiy the enzymes and other proteins that promote the various steps involved, we have designed an *in vitro* assay in which both the endonucleolytic cutting of damaged DNA and the induced 'cutting in *trans*' of undamaged DNA can be studied in crude extracts. This assay utilizes

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øX174 or ColE$_1$ Form I DNA as substrate and crude extracts made from thermally induced E. coli cells lysogenic for λ prec A$^+$, a transducing phage isolated by K. McEntee (5).

Methods. Bacterial strains were either recA$^+$ uvr$^+$, recA$^+$ uvrB$^-$ (both made lysogenic for λ precA cI857 S7) or recA$^-$ uvr$^+$ (made lysogenic for λ cI857 S7). In addition, they all carried recB sbcB endA to reduce the endonuclease I and exonuclease I and V activities. These strains will be referred to as wild type (wt), uvrB$^-$ and recA$^-$. Extracts were prepared as described by Gottesman, et al. (6) after thermal induction of these lysogens. Conditions for crosslinking DNA with trimethylpsoralen have been reported previously (7). Typical reaction mixtures (0.04 ml) contained approximately 50 μg/ml of DNA, and 6 mg/ml of protein extract. Incubations were at 37° for 90 min to 4 hours followed by centrifugation in alkaline sucrose gradients.

In all figures, sedimentation is from right to left.

RESULTS

Cutting in Control and Damaged Covalent Circular DNA.

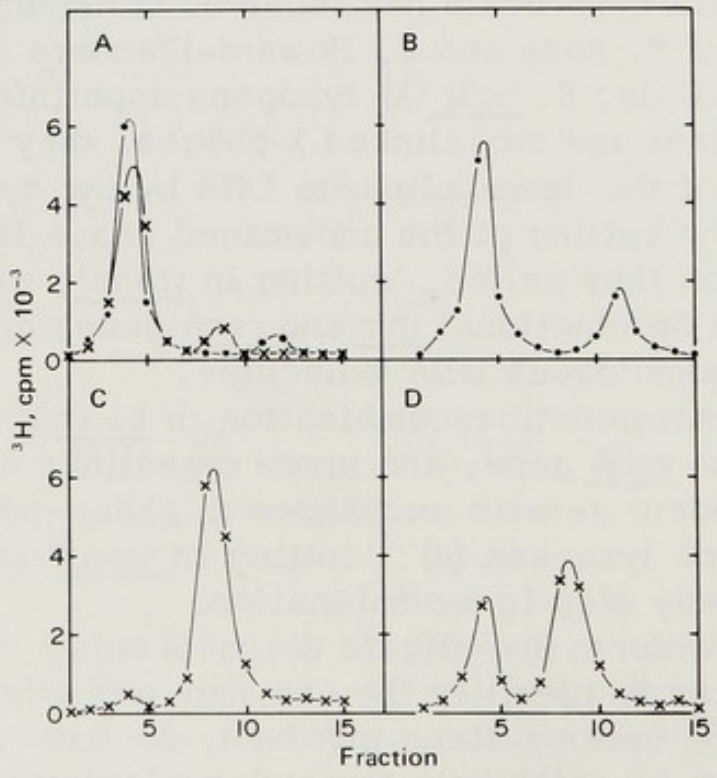

FIGURE 1. Endonucleolytic cutting of damaged DNA in crude extracts of E. coli. ●——● undamaged øX RF I DNA; x——x crosslinked øX RF I DNA. A: no incubation, B: incubation with extracts from wt, uvrB$^-$ or recA$^-$ cells, C: incubation with extracts from wild type or recA$^-$ cells, D: incubation with extracts from uvrB$^-$ cells.

^{3}H-øX RF I DNA contained initially about 90% of covalent circular form (Form I), as judged by sedimentation in alkali.

The addition of extract without incubation did not affect the sedimentation properties of intact or crosslinked ^{3}H-RF I DNA (Fig. 1A). After incubation for 60 min at 37°, 75% or more of the intact DNA was still in Form I (Fig. 1B). In contrast, damaged RF I DNA was cut when incubated with extracts of uvrB^{+} cells and sedimented at an intermediate speed (fractions 8-9, Fig. 1C). A limited amount of cutting was observed with extracts of cells carrying uvrB5 (Fig. 1D). This particular strain was subsequently found to be leaky for the uvrB$_5$ allele in in vivo experiments.

Interaction Between Crosslinked and Undamaged DNA Molecules. In order to detect the cutting of undamaged molecules induced by DNA containing partially excised crosslinks (cutting in trans), we incubated undamaged ^{32}P RF I DNA and either intact or crosslinked ^{3}H RF I DNA with extracts of wild type E. coli cells. Cutting in trans would be observed as a

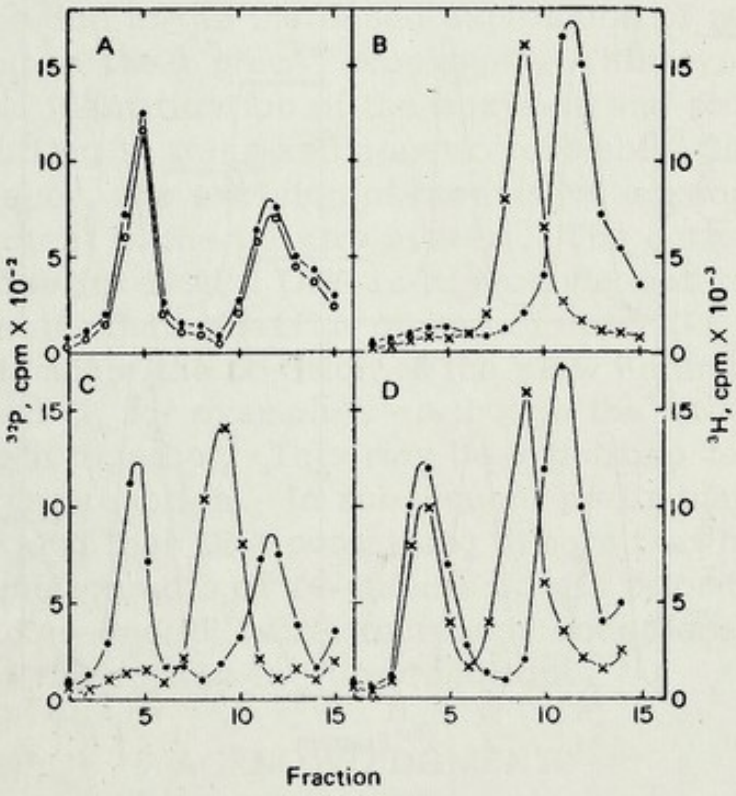

FIGURE 2. Cutting in trans induced by crosslinks: dependency upon recA$^{\pm}$ and uvr^{+}. ●——● intact ^{32}P øX RF I: x——x crosslinked ^{3}H øX RF I. Incubation with extracts from: A and B, wild type cells; C, recA^{-} cells; D, uvrB^{-} cells.

loss of ^{32}P-label in the fast sedimenting peak in alkaline sucrose gradients. Attempts to observe cutting in trans with extracts from nonlysogenic wild type cells failed. However, induction of wild type lysogens carrying λ precA$^+$ cI857 S7 prophages allows considerable amplification of the recA$^+$ gene product (8) and extracts made from these lysogens yielded the results reported below. Fig. 2 shows the yield of rapidly sedimenting ^{32}P DNA after incubation with control and damaged ^3H RF I DNA in an extract of wild type E. coli (λ precA$^+$ cI857 S7) cells. The yield of cuts made in trans was determined from the ratio of the percentages of ^{32}P DNA remaining in covalent circular form after incubation with crosslinked ^3H RF I DNA (Fig. 2B) and with control ^3H RF I DNA (Fig. 2A). By the same criterion, no cutting in trans was observed after incubation with an extract made from thermally induced recA$^-$ (λ cI857 S7) cells (Fig. 2C), and only a small amount of cutting was seen with an extract from uvrB$^-$ (λ precA$^+$ cI857 S7) cells (Fig. 2D).

Requirement for Homology. In order to test whether cutting in trans required genetic homology between the DNA

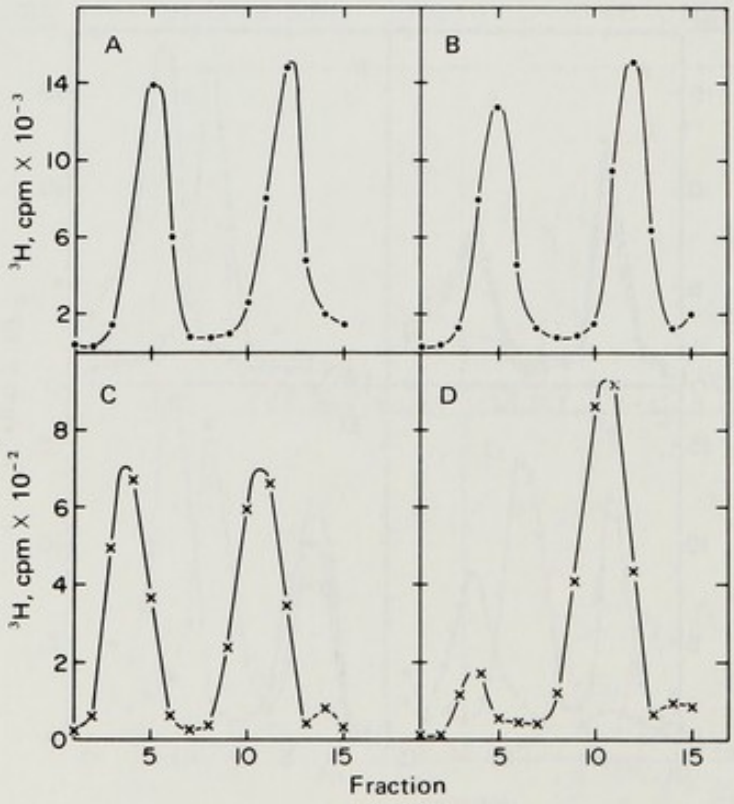

FIGURE 3. Cutting in trans in extracts from wild type cells: Requirement for homology. ●——● ^3H øX RF I; x——x ^3H Col E1 RF I. A and C: incubated with intact unlabeled Col E1 DNA; B and D: incubated with crosslinked unlabeled Col E1 DNA.

species involved, we performed a similar experiment in which one of the øX DNAs was replaced by the same amount of Col E1 DNA. Although we found no relevant data in the literature, we assumed that a large extent of homology between these two genomes was unlikely. Figure 3A and B shows that cutting in trans of intact øX RF I DNA occurred only to a very limited extent when incubated with crosslinked Col E1 DNA. In a further experiment (Fig. 3C and D), we showed that cutting in trans of intact Col E1 DNA incubated with crosslinked Col E1 DNA was similar to that seen in the øX system (compare Fig. 3C, D and Fig. 2A, B).

DISCUSSION

The system described here lends itself to further investigation. The results obtained so far are similar to those observed in vivo with phage λ infecting homoimmune E. coli lysogens. They provide preliminary evidence for an endonuclease activity that cuts intact superhelical DNA in response to interaction with homologous damaged DNA. Extracts made from thermally induced λ $precA^+$ lysogens of E. coli have proven to be substantially more active than extracts from non-lysogenic cells of the same strain. The increased activity appears to be due to the increased expression of recA (or other genes carried on the λ $precA^+$ prophage). This system may be useful for the identification of the enzymes and proteins required for cutting in trans and genetic recombination.

However, the excision of crosslinks appears to be rather inefficient in the in vitro system. The action of the extracts on crosslinked RF I DNA is to produce material that sediments faster than linear or nicked circular (RF II) undamaged DNA (compare the position of the slow sedimenting peak in Fig. 1B and C, for example), as though the DNA strands were still held together. This may be a limiting factor for the cutting in trans reaction. In subsequent preliminary experiments, we found that DNA containing dimers that had been excised by crude extracts of T4-infected cells prior to incubation with undamaged RF I DNA increased considerably the efficiency of the cutting in trans reaction.

ACKNOWLEDGMENTS

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THE INITIATION OF GENETIC RECOMBINATION IN *E. coli*: STUDIES ON CUTTING *in trans* AS A FUNCTION OF TIME[1]

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ABSTRACT Recombination enzymes are thought to act by cutting DNA molecules and joining them in register. Treatment with crosslinking agents can greatly increase the frequency of recombination. Experiments were performed with *E. coli* to biophysically detect the cutting of induced recombination. Phage λ DNA in *E. coli* (λ) lysogens was cut 'in *trans*' if superinfecting λ phages contained incised psoralen crosslinks in their DNA. Cutting in *trans* behaved as expected of a step in damage-induced recombination mediated by the bacterial enzymes. The present studies were undertaken to assess the amount of time required for cutting in *trans* under normal conditions and whether undamaged molecules that were cut in *trans* would be repaired on further incubation. Wild type cells, *recA*, *recF* and *lexA* mutants were tested.

The frequency of genetic recombination in bacteria may be dramatically increased by conditions favoring the formation of single-strand gaps in DNA molecules. For instance, irradiation with 254 nm light promotes recombination in replicating DNA, more effectively in *uvrA*$^-$ than in wild type cells, and the increase is attributed mostly to postreplication gaps formed in the damaged DNA.

Treatments (e.g. psoralen and 360 nm light) causing the formation of interstrand chemical crosslinks in DNA molecules, also cause recombination in bacteria. In λ phage-prophage genetic crosses under repressed conditions, psoralen damage in the DNA of infecting λ phages increased the frequency of recombination more than 50-fold. In this case, the damaged DNA did not undergo vegetative replication, and the endonuclease controlled by *uvrA*$^+$ was required for the increased recombination. Moreover, *recA*$^+$ was required, as expected if the enhanced recombination were mediated by bacterial enzymes rather than by those of the prophage or infecting phages (1).

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The above considerations, and certain convenient properties of the DNA from λ phages allowed to infect E. coli (λ), encouraged us to test for the initial cutting events in damage-induced recombination. An account of this work has been published (2).

When λ phages infect E. coli (λ) hosts, their linear DNA molecule is injected, and the ends are sealed together by polynucleotide ligase. The covalent circular DNA molecules so produced are readily detected following lysis, because they sediment through alkaline density gradients about four times more rapidly than other intracellular forms. First, we tested for a reduction in the proportion of λ DNA sedimenting rapidly if the infecting phages were damaged with psoralen. Psoralen damage in λ phages did not interfere with the injection or circularization of the phage DNA in the host cell, but damaged molecules were apparently cut by the cellular endonuclease controlled by $uvrA^+$. It was surprising, in view of the literature on DNA repair, that some of the cuts in the covalent circular molecules appeared to remain open at least four hours after infection with the damaged phages. This suggested the possibility that long-lived recombination intermediates were being formed.

Attempts were therefore made to detect cutting in undamaged DNA molecules if these were present in the same cell and so able to participate in recombination with the damaged molecules. When cells carrying nonreplicating covalent circular λ DNA molecules were superinfected with psoralen-damaged λ phages, the undamaged, covalent circular DNA was cut. This cleavage, referred to as 'cutting in trans', required both incision of the damaged DNA, and $recA^+$, but was unaffected by mutations in phage genes or by treatment with chloramphenicol. These results would be expected if the cutting were related to genetic recombination. Moreover, $recA^+$-dependent cutting in trans and damage-induced phage-prophage recombination were detected in experiments with the temperate phage 186, which showed no detectible base sequence homology or recombination with phage λ. However, in E. coli (λ) (186) double lysogens simultaneously infected with λ and 186 phages, both cutting in trans and damage-induced phage-prophage recombination were detected only in homologs of the damaged phages. The results indicate that intact DNA molecules paired with incised homologs, thus becoming substrates for a bacterial recombination endonuclease. $recA^+$ was required for pairing, for cutting in the undamaged duplex, or for both.

Cutting in trans as a function of time in wild type E. coli (λ): effect of varying amounts of damage and multiplicity of superinfecting phages. The time dependence of cutting in trans in wild type E. coli (λ ind) was investigated. Since the amount of damage and multiplicity of superinfecting phages might influence the time course of cutting in trans, these quantities were also varied.

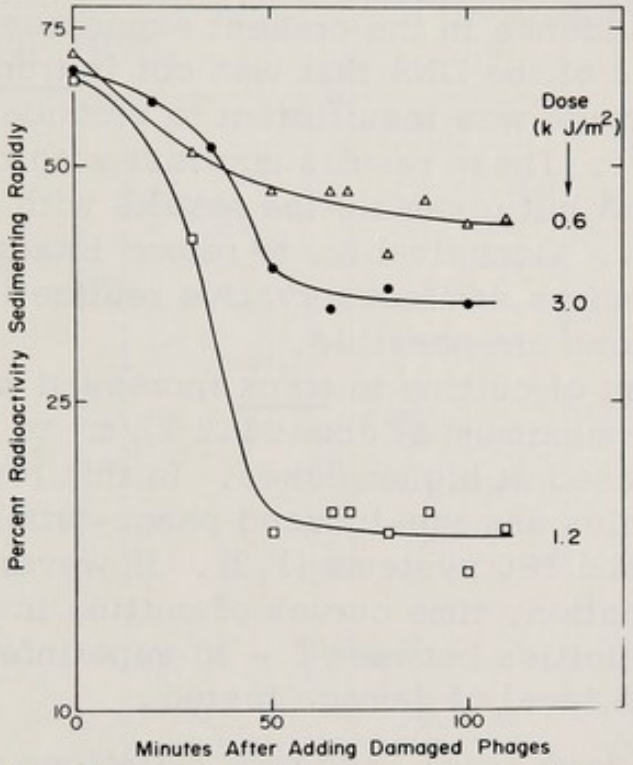

FIGURE 1. Percent radioactivity sedimenting rapidly in alkali from E. coli (λ) cells infected with undamaged ^{32}P-labeled λ phages as a function of time after superinfection with psoralen-damaged, unlabeled λ phages. Cells of wild type E. coli strain C600 (λ ind) were infected with two ^{32}P-labeled λ c71 per cell, incubated to permit circularization, and superinfected with psoralen-treated λ c71 phages irradiated as shown in Fig. 1. The cells were held 25 mins for adsorption, diluted in KM + 10 medium, and aerated at 37°C. Samples removed at various times after superinfection were lysed immediately. Procedures were previously described (2).

In Fig. 1, the proportion of ^{32}P radioactivity from lysates of mixedly infected E. coli (λ) that sedimented in alkali as λ covalent circles is plotted against the time of incubation between superinfection and lysis. When the superinfecting phages were exposed to 1.2 kJ/m^2 of 360 nm light, a dose producing about four crosslinks and 16 monoadducts per phage, about half of the undamaged covalent circles had been cut in trans within 35 mins after superinfection, while 75% of the covalent circles were cut in trans within 50 minutes.

Psoralen crosslinks in bacterial DNA were incised within a few minutes in competent cells (3). Molecular weight increases and density label exchanges in bacterial DNA containing postreplication gaps (4), or incised psoralen crosslinks (5), were detected about one hour after damage. Thus, cutting in trans was maximal following incubations apparently sufficient for recombination repair in bacterial DNA. However, there was no evidence in the present experiments for repair to covalent circles of the DNA that was cut in trans, even when the level of damage was insufficient to produce maximum cutting (0.6 kJ/m^2). These results contrast with those obtained on bacterial DNA but resemble the results with damaged, incised λ DNA (2). Conceivably, to reform intact DNA from DNA cut in trans requires semiconservative replication, though other explanations are possible.

The extent of cutting in trans increased with increasing irradiation to a maximum at about 1.2 kJ/m^2 of 360 nm light, and then decreased at higher doses. In this respect, cutting in trans resembles damage-induced phage-prophage recombination in both λ and 186 systems (1,2). However, unlike the induced recombination, time curves of cutting in trans did not vary for multiplicities between 5 - 30 superinfecting phages per cell at each level of damage tested.

Effect of lexA, recA, and recF mutations on cutting in trans. Recombination occurs in E. coli lexA1 at nearly the same frequency as in wild type cells (1). However, lexA mutants are partially defective in postreplication repair of ultraviolet-damaged DNA (6), indicating that recombination and conceivably other $recA^+$-dependent repair functions may be inefficient in these cells. The recF single mutant is somewhat radiation-sensitive and slow to fill postreplication gaps (6,7).

Experiments like those of Fig. 1 were performed in recA, in lexA, or in recF mutants of E. coli (λ ind), and in wild type cells. The results, summarized in Fig. 2, show that even at the high level of damage used in this experiment (3.0 kJ/m^2), cutting in trans could not be detected in the $recA^-$ hosts even after 110 minutes incubation in growth medium (Fig. 2a). Cutting in trans did occur in both $recF^-$ mutants (Fig. 2a, c) and $lexA^-$ mutants (Fig. 2b). However, a comparison of the curve for $recF^-$ with that for wild type controls (Fig. 2a) reveals that cutting in trans was slower in the mutant cells. Moreover, less cutting in trans took place in the $lexA^-$ mutants (Fig. 2b) than in wild type (Fig. 2a). The slowing due to $recF^-$ and reduction in magnitude due to $lexA^-$ of cutting in trans are

similar to the results with postreplication gaps (6,7). These genes may, therefore, affect not only rejoining, but also cutting in the second duplex during recombination. While no product of the recF or lexA genes has yet been identified, the low levels of recA$^+$ protein occurring in lexA cells may be insufficient for normal levels of cutting in trans, molecular weight increases in DNA containing postreplication gaps, and damage-induced recombination.

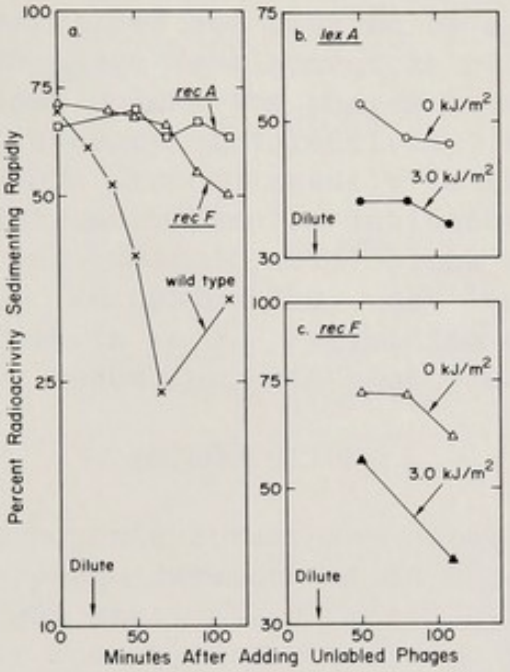

FIGURE 2. Host repair mutations affect the rate and extent of cutting in trans. Results are plotted as in Fig. 1. Procedures were as in Fig. 1.

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REPAIR AND RECOMBINATION OF UV-IRRADIATED PHAGE LAMBDA[1]

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ABSTRACT Intracellular λ DNA (from tandem duplication phages) was extracted and assayed by a transfection procedure. If phage development is blocked, recombination is very low, unless the phages have been UV-irradiated. Repair (recovery of infectivity) and recombination of irradiated DNA proceed readily in the presence of chloramphenicol and rifampin; inhibitors of DNA gyrase (coumermycin and oxolinic acid) block repair and reduce recombination. Recombination, very low in recA cells, is nearly normal in recB. Pyrimidine dimers seem not to be the primary recombinogenic photoproducts.

INTRODUCTION

For λ, recombinogenic structures arise during DNA replication. When phage development is blocked by repressor or antibiotics, λ DNA remains as "inert" closed circles, ideal probes for cellular recombination systems. However, repressed λ DNA is recombined poorly in phage-prophage crosses (1,2); we extend this conclusion to phage-phage crosses here. Assays which select against (unrecombined) monomer circles can yield high apparent recombination frequencies for inert λ DNA (3,4, 5).

Repair of damaged DNA provides highly recombinogenic substrates. Examples are those resulting from replication of UV-irradiated DNA (6), and from partial excision of psoralen cross-links (7).

A third class of repair-induced recombination, stimulated by unknown UV photoproducts in unreplicated DNA (2), is studied here, by an indirect method (Fig 1). The assay, which scores most of the DNA (including monomer circles), depends on intermolecular and intramolecular recombination (during the primary infection) between duplicated DNA regions (8,9). Recombinant "single-copy" DNA, when extracted and transfected into recArecB spheroplasts, yields EDTA-resistant phages. The procedure assays cellular recombination systems during the primary infection, when both cellular and phage-induced functions can be selectively inhibited.

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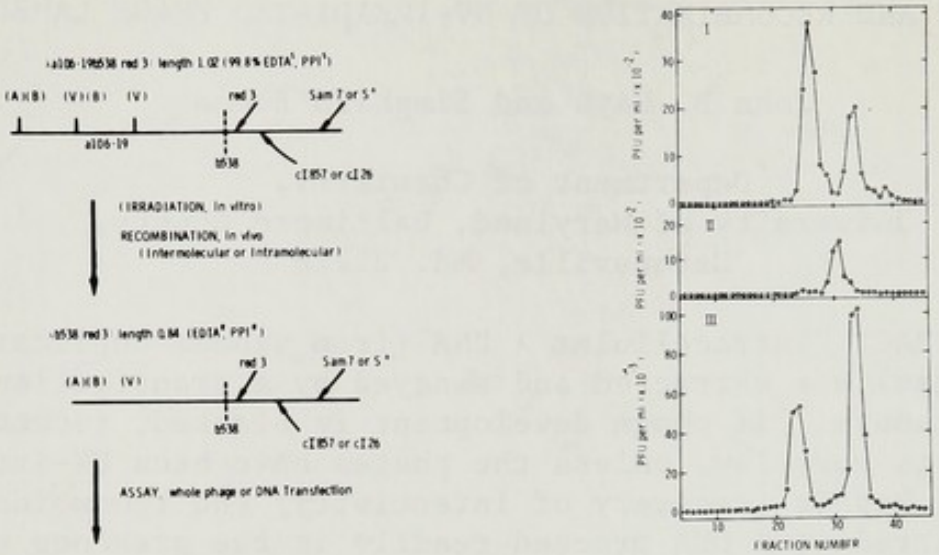

Figure 1. Transfection assay for recombination and repair. (A).Outline of procedure. Irradiation of λa106-19b538cI857red3 phage (germicidal lamp, 60-200 J/m^2), recombination in phage-infected cells (in presence of λ repressor and/or antibiotics, 20 phage/cell), DNA transfection assay: lysozyme-sarkosyl-Proteinase K lysis, phenol extraction and dialysis, transfection of recArecB spheroplasts, EDTA treatment, titer; recombination = [$EDTA^R$ phage]/[total phage]. (B).CsCl gradient analysis of typical transfection lysate.(I). Untreated. Recombination 30% by EDTA assay, 37% by relative peak areas.(II).EDTA-treated.(III).Mixture of lysates from control transfections (purified duplication and single-copy DNA).

RESULTS AND DISCUSSION

Recombination of "Inert" λ DNA. For lytic infections of rec^+ bacteria, the indirect transfection procedure gives the same result as direct assay of whole phage, 6% recombination after 90 min. For DNA in the presence of λ repressor, or chloramphenicol and/or rifampin, recombination is less than 0.1% above the recA background level.

Repair and Recombination of UV-irradiated λ DNA. The DNA from UV-irradiated phage is highly recombinogenic (50% recombination at 190 J/m^2), even in the presence of (ind^-) repressor or chloramphenicol and rifampin. Recombination increases with UV fluence; it is not affected by irradiation of the cells just before phage adsorption, and does not require de novo RNA or protein synthesis. The assay measures recombination, not UV-induced deletions; it is $recA^+$-dependent, and the recombined (EDTA-resistant) phage have the correct buoyant density (Fig 1B).

Coumermycin and oxolinic acid are drugs which act on

TABLE I
RECOMBINATION AFTER PHOTOREACTIVATION

Treatment[a]	Lytic Growth[b] Yield (PFU)	Recombination (%)	Repressed Growth[c] Time[d] (min)	Infectivity[e]	Recombination (%)
Dark	1.2×10^8	1.8	35,70	0.1,0.3	4.2,8.6
Light	4.1×10^8	2.7	35,70	0.7,1.6	1.2,1.1

[a] Cells [rec^+(λcI857red3xis1)] infected with UV-irradiated (140 J/m^2) λa106-19b538cI857red3 (30 phage/cell), shaken at 30° under visible light (two 15-watt fluorescent lamps at 3.2 cm, filtered thru 1-cm glass) or in dark for 30 min.

[b] Subsequent growth at 40°, chloroform lysis, EDTA assay.

[c] Subsequent growth at 30°, transfection-EDTA assay (Fig 1).

[d] Phage were mixed with cells at time zero.

[e] Infectivity of control DNA (2×10^{-4} PFU/equivalent) = 1.0.

different subunits of the E. coli DNA gyrase, an enzyme required for maintaining DNA supertwisting (10). Both antibiotics, at concentrations of 5-50 µg/ml, reduce repair of UV-irradiated (60-190 J/m^2) λ DNA by 5 to 20-fold; recombination frequencies are depressed 3 to 6-fold. A coumermycin resistant mutant is unaffected by the drug. Recombination may be inherently less gyrase-dependent, or there may be recombination pathways which do not require supertwisted DNA. The postulated role for supertwisting in recombination (11) could be a localized one; we observe substantial recombination (presumably intramolecular) at low multiplicities. The recombination frequency is very low in recA and recArecB cells, but nearly normal in recB infections, a result consistent with other reports of RecBC-independent recombination of λ (2,4).

In vivo photoreactivation increases the infectivity of heavily irradiated (140 J/m^2) λ DNA 5-fold, to approximately undamaged levels, but does not reduce the absolute number of recombinants (Table 1). Similar results have been reported for phage-prophage recombination (2). Although pyrimidine dimers appear to be ruled out, the nature of the recombinogenic photoproducts (and the mechanism of the repair-recombination processes) remain to be determined.

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XERODERMA PIGMENTOSUM

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ABSTRACT Xeroderma pigmentosum is an autosomal recessive skin disease in man, characterized by hypersensitivity of the skin to sunlight. Although the primary genetic defect in this syndrome has not yet been elicited, it is considered as being the best-studied DNA repair mutant in man. Heterogeneity is expressed at the clinical, genetical, biochemical and cellular level in this syndrome. It most probably includes a number of different genetic defects which all result in sensitivity of the patient's skin to sunlight.

INTRODUCTION

Since Cleaver's discovery in 1968 (1) of a defect in DNA repair in xeroderma pigmentosum (XP), this genetic skin disease has become the best-studied mutation in DNA repair in man. A rather complex picture has emerged from these studies.

At the clinical level, as in so many other genetic diseases, heterogeneity in the expression of symptoms is evident. Hypersensitivity of the skin to sunlight is observed in all patients. It is expressed in a dry, atrophic skin with marked pigmentation in the sun exposed areas. Most XP patients develop premalignant actinic keratoses followed by cutaneous neoplasms of different cellular origin. There is a broad spectrum of neurological defects in XP. A clinically well defined class of patients, having the most severe form of neurological abnormalities is the De Sanctis Cacchione Syndrome (DSC) (2). Only a few patients have this complete syndrome, others have one or more of these abnormalities and some patients seem to be free of neurological defects at all. A progressive mental deterioration is observed in many cases. This variable pathology of the disease makes a classification of XP patients in a DSC and classical form of XP (3) ra-

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ther unreliable. For review of the clinical features of XP the reader is referred to Robbins et al. (2) and Kraemer (4).

In biochemical and genetical studies heterogeneity has been observed at the molecular and cellular level. Biochemically two classes of XP patients can be identified: patients whose cells show a defective excision of DNA lesions induced by ultraviolet light (excision deficient XP) and patients whose cells show normal excision but impaired postreplication repair (XP variants) (5). Genetic studies have demonstrated the existence of different mutations affecting excision repair in the XP syndrome (2,3). Xeroderma pigmentosum, therefore represents a complex syndrome. It includes different genetic defects resulting in sensitivity to ultraviolet light (UVL) and other DNA damaging agents.

GENETICAL CLASSIFICATION OF XP

Excision deficient XP.

Excision repair is visualized autoradiographically by the incorporation of ^{3}H-thymidine in UVL exposed cells in the G1- or G2- phase of the cell cycle (unscheduled DNA synthesis, UDS). Excision deficient XP cells are characterized by decreased levels of UDS (1).

The tools for the genetic analysis of defective DNA repair in XP were provided by the development of cell fusion procedures. Cultured skin fibroblasts of different XP patients were fused and DNA repair was studied in hybrid multinucleated cells (heterokaryons) after exposure to UVL (3). In several combinations of excision deficient XP strains the heterokaryons showed normal levels of UDS. This restoration of DNA repair was interpreted as resulting from complementation between two different repair mutations present in the parental genomes.

In a joint paper the Rotterdam and Bethesda group, involved in these studies, described the presence of 5 different complementation groups (6): A, B, C, D and E. Recently in a large survey of Japanese XP patients, Dr. H. Takebe and coworkers at Kyoto found a patient, XP23OS, whose cells com-

plemented the A to E group cells resulting in complementation group F (7). In collaboration with Drs. A.M.R. Taylor and D.G. Harnden (Birmingham, England) we found the 7th complementation group in excision deficient XP, group G, represented by patient XP2BI (8).

These 7 complementation groups represent 7 different mutations affecting excision repair. The genes involved may be regulatory as well as structural genes. It is reasonable to assume that cell strains belonging to the same complementation group are defective in the same repair function. They might be mutated at different sites in the same gene (if intragenic complementation does not occur) or even in different genes (if the formation of "heteropolymeric molecules" is limited in the heterokaryons).

The classification of XP strains in complementation groups is mainly based on the determination of UDS. It has been suggested that cell strains belonging to the same complementation group are characterized by comparable levels of UDS (2, 9). The distribution of UDS levels over the complementation groups does not support it (10, 11). Although data obtained by different laboratories on the same XP cell strain are not always consistent (compl.group D; 2,11,12,13), the levels found in the A, C and D group by the same investigators clearly show extensive but reproducible differences. This variability might be expected, assuming the presence of mutations at different sites in the same gene (or even in different genes) affecting the protein molecule in different ways.

The detection of the new complementation groups F and G clearly demonstrates that assignment of new XP strains to complementation groups based on UDS levels and clinical symptoms only, is a misleading procedure. Following that strategy the G group strain would have been assigned to complementation group A.

XP Variants

Cultured skin fibroblasts of XP variants show a slow rate of postreplication repair. The conversion from low to high molecular weight DNA, which occurs following DNA replication after exposure to UVL, is inhibited by caffeine (5) in XP variant cells. Caffeine has little effect in normal human

fibroblasts. In heterokaryons obtained after fusion of XP variant cells with normal cells Dr. Jaspers in our institute found restoration of the postreplication repair defect in the variant nuclei of the multikaryons. In fusions between different variant cell strains complementation was not observed. These preliminary data indicate that the genetic basis of the defect in XP variant cell strains is less heterogeneous than in excision deficient XP (Jaspers, unpublished observations).

MODIFICATION OF THE GENETIC DEFECT

In attempts to modify the genetic defect(s) in excision deficient XP cells various approaches have been followed. Incubation with homogenates of normal cells, cocultivation with normal cells (14), and infection with high multiplicities of T4 phage (15) had no effect. Positive results were obtained in experiments performed by Tanaka et al. (16). UVL induced UDS in XP cells belonging to the groups A, B, C, D, and E was enhanced by pretreatment of the cells with inactivated Sendai virus and incubation with an endonuclease V preparation from T4 bacteriophage. In a recent publication (17) these authors showed that this treatment with T4 endonuclease increased the survival of group A cells after UVL exposure. The T4 enzyme is known to have a high specificity towards pyrimidine dimers. These interesting observations demonstrate the incapacity of group A, B, C, D and E XP cells to perform the incision step of the dimer excision process (although, because of the specificity of the T4 enzyme, a defect in an exonuclease step cannot be excluded with certainty).

BIOCHEMICAL CHARACTERISTICS OF XP CELLS

Excision deficient XP

In a large number of studies the effects of different DNA damaging agents on XP cells have been investigated. These agents generate a variety of substrates for repair enzymes. It is expected that some of these substrates produced by different agents are structurally related and require similar pathways for their repair. Consequently a genetic defect in repair might render the cells sensitive to the action of different agents. This might provide an explanation for the occurrence of neuro-

logical defects in XP patients. These defects could be tne result of defective repair of DNA lesions which occur spontaneously or have been produced by chemical agents in neuronal cells (4).

In this review the discussion mainly concerns recently obtained data on the repair of DNA lesions induced by UVL. For more extensive data on the repair of damage produced by ionizing radiation and chemical mutagens and carcinogens the reader is referred to Regan et al. (18) and Maher et al. (19).

A variety of observations mainly concerning XP cells of group A and C are in line with a defect in the incision step as suggested by the experiments of Tanaka et al. (16). A and C group cells show defective removal of thymine dimers (20,21,22) and new approaches in the study of UVL induced DNA strand breaks, probably reflecting incision steps of DNA repair, have indicated defective nicking in these cells (23,24). A conflicting result in this respect is the finding by Dingman et al. (25) of normal nicking but defective closure of breaks in A group cells following exposure to UVL. A problem in these experiments is provided by the possible action of degradative enzymes not specifically related to DNA repair. This degradation may result in decreased sedimentation rates of the DNA (26).

Dimer removal has not been described for the other XP complementation groups. The new test systems for measuring incision induced strand breakage have been applied to B (23) and D (23,24) group cells and showed defective nicking in these XP strains as well.

A surprising result concerning the dimer specific incision was obtained in studies of unfractionated extracts of XP cells by Mortelmans et al.(27). Thymine dimers present in UVL exposed E.coli DNA were excised at the same rate by extracts of normal cells and XP cells of group A, C and D. In earlier studies Cook et al. (28) have shown that extracts of group A and D cells were able to excise thymine dimers from E.coli DNA which was previously incised by a dimer specific T4 phage endonuclease. Apparently these XP cells posses endo- as well as 5' - 3' exo-nucleolytic activity required for the excision of thymine dimers. The finding of normal dimer specific endonuclease activity in group A and C cells seems to be in conflict with defective nicking and dimer excision demonstrated di-

rectly in these cells. A clue to this problem was provided by the observation of defective excision of the dimers from the chromatin present in the extracts of A group XP cells. Apparently this defect is not at the level of the substrate, since extracts of normal cells were able to excise dimers from XP chromatin. The authors suggest that the excision of dimers in unfractionated cell-free extracts requires an additional factor which is defective in extracts of A group XP cells. This "XP-factor" might act in close association with dimer-specific endonuclease. Purification and further characterization of this factor in these and the other XP complementation groups is in progress. These studies clearly indicate that eukaryotic cells,because of the complicated structure of chromatin, require additional steps in the repair process in order to facilitate the interaction of the repair enzymes with the chromosomal DNA.

Thymine dimers are probably not the only UVL induced lesions whose repair is reflected in UDS as a result of the replacement of a relatively large number of nucleotides (long patch repair). The lack of dimer excision in the presence of UDS in chicken fibroblasts (29) points to the possible contribution of the excision of other lesions to the ultimate level of UDS observed in eukaryotic cells. Defective excision of DNA/protein crosslinks in group A cells, which is inferred from alkaline elution experiments (30) indicate that the XP factor also plays a role in the repair of these lesions.

A minor class of UVL induced lesions are base damages of the 5,6-dihydroxy-dihydrothymine (t') type. Hariharan and Cerutti (31) presented evidence for the presence of normal endonucleolytic activity recognizing t' lesions in nuclear preparations of A, C and D group XP cells. Their experiments do not rule out the possibility of "XP factors" required for the action on t' lesions in DNA packed in chromatin.

Damage involving more than one nucleotide, which might cause major helix distortions of the DNA molecule like pyrimidine dimers, are also produced by ionizing radiation and alkylating agents. In the case of ionizing radiation the nature of these lesions is not completely characterized. DNA interstrand and DNA protein crosslinks can be considered as lesions of this type. Little is known about the repair of these lesions in mammalian cells. Studies

on cells of patients suffering from the genetic disease ataxia telangiectasia, which probably have a defect in the repair of γ-rays damage, will be most helpful in this respect (see review of Paterson in this symposium).

In a recent study Setlow et al.(32) described a defective repair of some rather unidentified lesions following exposure of XP cells (of unknown complementation group) to ionizing radiation. The defect is only observed after irradiation under anoxic conditions and involves a repair pathway which mimics "long patch repair" following exposure to UVL.

A rather unexpected result regarding the repair of mitomycin C induced interstrand crosslinks has recently been published by Fujiwara et al. (33). Group A cells were found to repair interstrand crosslinks at a normal rate. The pathway involved in the repair of this lesion was clearly different from the dimer excision pathway in these cells. The same study showed that the repair of monoadducts (damage involving only one base) produced by monofunctional decarbamoyl mitomycin C was impaired in these A group cells. The defect in the A group is also expressed in the defective removal of adducts produced by 4-nitroquinoline 1-oxide (4NQO) (34) and N-acetoxy-2-acetylaminofluorene (AAAF) (35). Concerning AAAF damage Ahmed et al. (36) and Amacher et al. (37) presented evidence that the repair pathway is different from the repair of UVL induced damage. It seems likely that the A (and also the C (35) group mutation affects both repair pathways.

The influence of one genetic defect (e.g. the mutation in group A) in the repair of a number of different lesions can be explained in different ways. The pathways involved in the repair of these lesions may have one or more steps in common, one being deficient in A group cells. It is also possible that the enzymatic pathways are different but the expression of a number of related repair processes is under similar genetic control.The A group cells may be deficient in this control function. The *in vivo* correction experiments of Tanaka (16) and the XP factor postulated by Mortelmans et al. (27) would then indicate that this control function is closely associated with the endonucleolytic step and does not affect later functions in excision repair.

Consistent with a defect in a control function is the observation of defects in two other repair processes in XP cells, the repair of apurinic sites and photoreactivation. Recently Kuhnlein et al.(38) described altered characteristics of apurinic endonuclease in extracts of D group, and to a lesser extent also of A group cells (see ref. 39 for a discussion of the pathways involved in the repair of apurinic sites). Following the detection of photoreactivation in human cells (40), Sutherland et al. described impaired photoreactivation in A, B, C, D and E group cells (41). Difficulties in reproducing these results in other laboratories (42) indicate that the demonstration of photoreactivating activity in human cells is very sensitive to still unidentified factors in the medium. Further work on the nature of this photoreactivation system and its biological relevance is underway and will hopefully solve the present uncertainties.

XP Variants

The work of Lehmann et al. (5) has elicited a postreplication repair defect in excision proficient XP cell strains (XP variants). At the molecular level this repair mechanism is not yet fully understood. It presents itself as a bypass mechanism allowing DNA replication to pass DNA lesions present in the parental DNA strand. Gaps occurring in the daughter strains are rapidly sealed in normal cells, whereas this sealing process seems to be inhibited in XP variant cells. This difference between variant and normal cells is enhanced in the presence of caffeine (see for review ref. 43). XP variant cells have been tested in many of the studies discussed in the previous chapter (23, 25, 30, 38). Evidence is presented for a slower sealing of UVL induced strand breaks in variant cells compared to normal cells (23,25). This would imply that in addition to delayed sealing of gaps in newly synthesized DNA (5) also repair of parental DNA is impaired. Fornace et al.(23) attributed this impairment to difficulties in the repair of dimers if DNA repair synthesis encounters a dimer in the template.

Recently Friedberg's group (44) found defective excision of dimers from the chromatin in extracts of XP variant cells. Although dimer excision is not inhibited in intact variant cells, these

data might indicate that excision repair and post-replication repair have some steps in common, or may be under similar genetic control. Consistent with this assumption is the observation of excision repair deficient XP cells having postreplication repair patterns which are intermediate between variant and control cells (5).

CELLULAR ASPECTS OF XERODERMA PIGMENTOSUM

The repair defect in XP is expressed at the cellular level in different ways. An increased sensitivity to ultraviolet light is indicated by the colony forming ability of XP cells. The sensitivity varies with different complementation groups (see for review ref. 4). This increased sensitivity is also observed for XP cells which have been treated with compounds that generate UDS in normal cells and not in XP cells (NQO, AAAF and others). Normal survival is observed following ionizing radiation and exposure to chemicals which generates normal levels of UDS in XP cells (methylmethane sulfonate, MMS and N-methyl-N'-nitro-N-nitrosoguanine)(see ref. 4, 18, 19).

Andrews et al. (45) compared the UVL sensitivity of cells from XP patients having neurological abnormalities with those of patients having only skin lesions. They found that the most sensitive strains were derived from patients with mental defects (most of the A group and all D group strains). This sensitivity pattern does not correlate with UDS levels found in these cells. However, this increased sensitivity of A and D group cell strains from patients with neurological defects may be associated with the altered apurinic endonuclease found in these cells by Kuhnlein et al. (38). Analysis of apurinic endonuclease in A group strains from patients having only skin lesions (XP1LO and XP8LO) could be informative in this respect. It is tempting to postulate that defective repair of spontaneously formed apurinic sites is responsible for the neurological degeneration observed in these group A and D patients (38). Possibly connected with this defect is also the observation that host cell reactivation, measured as survival of UVL exposed adenovirus 2 (46) and SV40 virus DNA (47) is most severely affected if A and D group cells were used as hosts.

The survival of XP variant cells is either indistinguishable from normal (48) or shows a slight increase in sensitivity after exposure to UVL (19, 49). The sensitivity of these cells is also expressed in decreased host cell reactivation (46,47). The small differences between XP variant and normal cells in these survival studies indicate that the genetic defect(s) in XP variant cells are less important for the survival of the cells than are the defects in the excision deficient XP cells. Clinically, however, all forms of XP show similar skin symptoms including the high frequency of tumor formation. A clue to this problem might be the increase in UVL induced mutation frequencies observed in all forms of XP in comparison to normal cells (19), which support a possible association of mutagenesis and carcinogenesis.

Further studies on the nature of the genetic defects in the different forms of XP and their role in mutagenesis and carcinogenesis are in order. These studies will include the isolation and purification of enzymes and other proteins("XP factors") involved in DNA repair. The picture emerging from 10 years xeroderma research clearly demonstrates the complexity of these problems which is likely to be associated with the higher organization of the genome in eukaryotic cells.

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WORKSHOP SUMMARY: XERODERMA PIGMENTOSUM

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Ten years ago xeroderma pigmentosum (XP) became the first human disease in which DNA repair processes were found to be defective. This autosomal recessive disease is characterized in all patients by accelerated chronic solar degeneration of sun-exposed skin and, in some patients, also by neurological abnormalities (NA). In the past decade studies of XP cells have contributed considerable information concerning human DNA repair processes. We are now beginning to understand the role of some of these repair processes in carcinogenesis, mutagenesis, and gene conservation. Since defects in specific DNA repair processes in XP patients result in certain well-defined clinical abnormalities, we can now ascribe the absence, or at least the delayed onset, of such abnormalities in otherwise healthy humans to the proper functioning of these repair processes.

Progress in understanding human DNA repair and its relation to human disease could not have been made were it not for the previous years of research on the DNA repair, nucleic acid biochemistry, photophysiology, and genetics of non-human cells and, particularly, of prokaryotic organisms. The study of XP, therefore, represents an outstanding example of the successful application of 'basic research' to clinical manifestations of human disease. This XP workshop has focused attention primarily on describing how defective human DNA repair processes are related to premature death of neurons and to increased susceptibility of skin to UV-induced damage, including tumor formation. In order to understand these relationships it was necessary to describe certain aspects of the structure and physiology of the skin and of the central nervous system as well as the important relevant clinical features of XP and of Cockayne's syndrome. This information is necessary to choose appropriate cell strains for study and to relate experimental results to the pathophysiology of clinical abnormalities.

Dr. John Epstein has reviewed in this workshop the nature of human skin cancers which are induced by the ultraviolet (UV) rays from the sun. A similar spectrum of such cancers occurs in XP patients but at an earlier age and with a greater frequency than in normal humans. He has described

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some of the complex acute effects of UV in mammalian skin. These effects include production of photochemical damage in DNA, inhibition of mitosis, and disruption of synthesis of DNA, RNA and protein. Possible relationships between these acute effects and UV carcinogenesis are still obscure. One of the most important of the chronic effects of UV on mammalian skin is the formation of cutaneous malignancies. Using the hairless mouse model, Dr. Epstein has been able to study pathological and other features in the development of invasive squamous cell skin cancer. These experiments suggest that UV carcinogenesis starts with the first exposure. Furthermore, the progressive pathophysiologic steps in mouse skin appear very similar to those found in human skin, progressing from benign hyperplasia to premalignancy and then to frank cancer. Even though the effect of UV radiation may be to produce photochemical damage in DNA which eventually leads to a mutation(s) resulting in a cancer, the events occurring between that initial damage and the development of the cancer are not fully understood. The mouse system permits more detailed and controlled studies of these events than can be performed in humans and such studies promise to elucidate the physiology, pathology, and biochemistry of UV-induced cutaneous tumors in humans.

Dr. Takebe has presented very extensive studies of some of the clinical manifestations and DNA repair characteristics of XP patients in Japan. The complementation group most frequently found among Japanese patients is group A, and, so far, no patient belonging to group C has been identified. These results are in striking contrast to findings in the United States and in Europe where the most common group appears to be group C. Most of these Japanese group A patients are children who have NA and low rates of UV-induced unscheduled DNA synthesis (UDS) (< 10% of the normal rate). These features are present in most, but not all, group A patients in the United States and Europe. XP variants have also been identified in Japan. Dr. Takebe described a very unusual patient, XP23OS. This patient had no NA and had 'moderate' skin lesions. Cells from XP23OS had UV-induced UDS levels of 10% of the normal rate and had higher host-cell-reactivation capacity for UV-irradiated herpes simplex virus than did cells from groups A, B, C and D. Cell fusion studies indicate that XP23OS belongs to a new complementation group designated group F.

Dr. Andrews and I have reported on the correlation between the post-UV colony-forming ability (CFA) of XP fibroblasts and the age of onset of the patients' NA. These are the first studies to show a relationship between DNA repair and the NA of XP. Clinical and autopsy studies

indicate that these XP NA are due to premature death of neurons in the absence of otherwise recognizable and specific histopathology. Fibroblasts from XP patients with an early onset of numerous XP NA had the lowest post-UV CFA, while patients with a later onset had greater post-UV CFA. Patients with only a few NA during their second decade of life had still greater post-UV CFA, and the patients without NA had the greatest post-UV CFA. We consider these differences in ability of XP strains to form colonies after UV irradiation to reflect the different inherited capacities of the cells to repair their damaged DNA to the biologically functional level required to complete repeated cycles of cell division. These results suggest that different kindreds within a single complementation group (e.g., A or C) display genetic polymorphism by having different inherited mutations in their DNA repair gene product. The results also suggest that XP patients with NA have DNA repair processes which are so defective that neuronal DNA, perhaps damaged 'spontaneously' or by endogenous chemicals, is not repaired to the extent required for neuron survival. Such damaged neuronal DNA apparently requires the same process(es) for its repair as DNA damaged by UV radiation.

All the work we have presented concerning XP post-UV CFA has been conducted with fibroblast strains. Decreased post-UV CFA of the fibroblasts can be considered a form of cell death, death being defined as the inability to divide and form colonies. If the differing inherited DNA repair capacities of these cells are responsible for their differing post-UV CFAs, the same relative differences in post-UV survival should be demonstrable in other cell types from these patients. Dr. Andrews and I wished to see if the same relative sensitivities to UV shown by XP fibroblasts in our post-UV CFA experiments could be demonstrated with a slightly different test of cell killing and with a different cell type. Accordingly, we prepared lymphocyte cell lines (LCLs) from many of our XP patients. LCLs are Epstein-Barr virus-transformed B lymphocytes. The transformed LCLs grow in suspension and are immortal. We had previously shown that XP patients' LCLs manifested the same DNA repair-dependent abnormalities as their fibroblasts, i.e., decreased UV-induced tritiated thymidine incorporation and increased UV-induced sister chromatid exchanges. We measured LCLs' survival by their ability to exclude trypan blue on the fourth day after a UV dose which did not decrease the survival of normal control donor LCLs. All the XP patients' LCLs whose survival could be accurately quantitated had the same relative survival as their fibroblasts (cf. Fig. 1 of Andrews, A. D., Barrett, S. F., and Robbins, J. H., this workshop). The relative survival of the LCLs tested was as follows:

normal control donors' LCLs > group E (XP3RO) > XP variant (XPPHBE) > high group C (XP2BE; XP8BE; XP9BE) > low group C (XP1BE, XP3BE) ≥ group D (XP7BE; XP17BE) = group A (XP12BE). Any differences in survival among the LCLs of the low group C patients and those of the group D and A patients were obscured by the low survival of these LCLs at the UV dose used in these experiments, and these LCLs will be retested with lower doses in an attempt to detect any differences which might exist among them.

Fibroblasts from patients with the autosomal recessive disease Cockayne's syndrome (CS) also have decreased post-UV CFA. This sensitivity to UV radiation may be due to a DNA repair defect, but further studies will be required to determine this. CS patients have NA which differ from those of XP patients; however, CS NA result from death of nerve, Schwann, leptomeningeal and other cells, and this death could be caused by an endogenous chemical(s) which kills CS cells by virtue of the same mechanism as that involved in the killing of CS fibroblasts *in vitro* by UV radiation. CS patients, with the exception of XP11BE who has both XP and CS, do not develop abnormal freckling or excessive numbers of tumors on sun-exposed skin. It is therefore possible that CS cells may have a frequency of mutation to UV radiation equal to, or lower than, that of normal human cells. CS cells with a lower mutation frequency could have a defect in a process required for establishing and/or propagating UV-induced somatic mutations.

Since most neurons in the human central nervous system lose the capacity to undergo mitosis by the time of birth, and virtually all the remaining neurons lose that capacity by the end of the second postnatal year, postnatal neurons are considered 'post-mitotic.' Such post-mitotic neurons may not have functional excision repair even in normal humans, since other post-mitotic mammalian cells are known to lose this capacity. Therefore, it may be that XP neurons accumulate more unrepaired DNA damage *in utero* than neurons of normal fetuses. In postnatal life additional DNA damage occurs which is not repaired in either XP or normal neurons. This damage, added to the excessive amount already present at birth, would therefore impair survival of XP neurons at a relatively early age. The hypothesis that *in utero* accumulation of damage in XP neuronal DNA leads to very early postnatal, and even to *in utero*, death of neurons is supported by the presence of severe microcephaly in those XP patients whose fibroblasts are most easily killed by UV radiation as measured by our post-UV CFA test.

The demonstration that defective DNA repair processes in XP can cause premature death of neurons indicates that

normal DNA repair processes are required to ensure the functional integrity of the DNA of the nervous system. Even though neuronal DNA does not replicate in post-mitotic neurons, the functional integrity of that DNA is essential for the vital transcription of RNA. Therefore, damaged neuronal DNA could be involved in the pathogenesis of other degenerative diseases of the human nervous system as well as in 'normal aging' of that system. The loss of functional integrity of neuronal DNA can be the result of inherited defects in DNA repair processes such as in XP and other diseases which may have inherited DNA repair defects, e.g., ataxia telangiectasia, Fanconi's anemia, and Cockayne's syndrome. Alternatively, neuronal DNA could be excessively damaged in the absence of inherited defects in DNA repair by excessive quantities of endogenous or exogenous agents which damage DNA or inhibit DNA repair processes. Such agents could be concentrated in particular parts of the nervous system and could result from normal or abnormal metabolism, or they could be exogenously acquired factors such as slow viruses, aldehydes derived from alcohols, anti-cancer drugs, or other toxic or mutagenic chemicals.

DNA REPAIR NUCLEOTIDE SEQUENCES WHICH PREVENT PREMATURE DEATH OF NEURONS IN HUMANS: EVIDENCE FROM STUDIES ON XERODERMA PIGMENTOSUM

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ABSTRACT Evaluation of xeroderma pigmentosum patients' clinical presentations, complementation group assignments, and post-ultraviolet irradiation colony-forming abilities indicates that, among DNA nucleotide sequences which affect DNA excision repair, there are at least two, and possibly three, discrete sequences which are required to prevent premature death of nerve cells. These three sequences define complementation groups A, D, and C, respectively.

INTRODUCTION

Fibroblast strains from xeroderma pigmentosum (XP) patients who have an early onset of numerous neurological abnormalities (NA) (some group A patients) have the lowest post-ultraviolet (UV) irradiation colony-forming ability (CFA) among XP strains (1-3). Less sensitive strains are from patients with a later onset of these NA (group D patients), while the most resistant strains are from patients without any NA (1-3). We have applied our post-UV CFA test to strains from two XP patients who have so few NA that it was not possible clinically to determine whether the patients' NA were, in fact, related to their XP or simply coincidental. Complementation group assignments of these patients and the levels of their UV-induced unscheduled DNA synthesis (UDS) were also determined.

METHODS

The patients' post-UV CFA was a measure of their fibroblast strains' colony-forming efficiency retained after irradiation with 254 nm UV light (1-3). Complementation groups

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were determined by autoradiographic studies of the rate of UV-induced UDS in heterokaryons (4, 5) formed by fusing XP fibroblast strains from the various complementation groups (4-6) with strains from the two patients who had only a few NA. One patient, a 17-year-old woman reported to us to have mental retardation and microcephaly, had fibroblast strains CRL 1333 and GM 2096 established from separate skin biopsies at the American Type Culture Collection and at The Human Genetic Mutant Cell Repository, respectively. All experimental results with strain CRL 1333 were identical with those obtained with strain GM 2096. The other patient, a 12-year-old girl whom we find to have only areflexia and an abnormal electroencephalogram, had strain XP12BE established from a biopsy obtained when she was 7 years old (4) and has previously been assigned to group A (4-6) and found to have the typical group A rate of UV-induced UDS which is 0.4 to 1.3% of the normal rate (7). The rate of UV-induced UDS was determined autoradiographically (4, 5).

RESULTS

The post-UV CFA of XP12BE and of GM 2096 was found to be within a post-UV CFA "transition zone," that is, their post-UV CFA was greater than the post-UV CFA of XP patients with numerous NA but less than that of patients with no NA. This transition zone is bounded, therefore, along its lower portion by the highest group D post-UV CFA curve and along its upper portion by the lowest group C post-UV CFA curve (Figure 1 in Andrews, A.D., Barrett, S.F., and Robbins, J.H., this Volume). Complementation group determination revealed that GM 2096 was in group C, because fusing her cells with either group A, D, or E cells resulted in fully restored UV-induced UDS in the nuclei of the heterokaryons, while fusion with group C cells did not restore any UV-induced UDS. GM 2096 was found to have the typical group C rate of UV-induced UDS, which is 10 to 20% of the normal rate (5).

DISCUSSION

Sufficient numbers of the characteristic NA of XP have been found in each of several group A and group D patients (1-4) to indicate that the nucleotide sequences defining the groups A and D, respectively, determine the occurrence of the NA of XP in these patients, all of whom have very low post-UV CFA (1-3). There is considerable evidence to suggest that the nucleotide sequence defined by the group A defects and the sequence defined by the group D defect(s) are distinct and do

not overlap, since fusing a group A fibroblast with a group D fibroblast results in a heterokaryon with apparently fully restored UV-induced UDS (4-6) and host-cell-reactivation (8).

Since the post-UV CFA transition zone is situated above the neurological group D zone but below the zone formed by the non-neurological group C patients' cells, we might expect that at least some XP patients whose post-UV CFA is within this transition zone would have only a few clinically apparent NA of XP in their second decade of life. This expectation seems to have been fulfilled by the 12-year-old group A patient and by the 17-year-old group C patient. It is likely, therefore, that the NA of these patients are related to, and caused by, their XP. Since autoradiographic complementation group analysis revealed that this group C patient's cells restored a perfectly normal rate of UV-induced UDS to group A and to group D cells upon fusion with them, the possibility is very unlikely that these group C cells contained, in addition to their defect in the group C nucleotide sequence, a defect extending also into any part of the nucleotide sequences defining either group A or group D. If these speculations are correct, we can conclude that the DNA nucleotide sequence which defines group C, as well as the sequences which define groups A and D, must effect a certain level of functionally adequate DNA repair to prevent premature death of neurons in normal humans.

Currently, the only other XP patient whose post-UV CFA we have found in the transition zone is XP11BE. This patient is unique among XP patients: she is the only patient known to be in group B, and her fibroblasts complement those from every other group (4-6); her UV-induced UDS is 3 to 7% of normal (4, 5); and she has (4) another rare autosomal recessive disease, Cockayne's syndrome (CS). CS is characterized by acute sun sensitivity, cachectic dwarfism, neurological and skeletal abnormalities (9-11). CS patients, however, unlike XP11BE, do not develop tumors or abnormal freckling on sun-exposed skin (11). We have studied fibroblast strains from nine CS patients and found all nine strains to have decreased post-UV CFA but normal rates of UV-induced UDS (12). The post-UV CFA of XP11BE, however, is lower than that of any of the nine CS strains. We attribute her more marked UV sensitivity to a summation of the sensitivities derived from both her XP and CS defects. When allowance is made for the decreased post-UV CFA shown by all the CS patients who have no coincident XP, we can attribute only a relatively small amount of decreased post-UV CFA to XP11BE's XP. These results and analyses are in accord with our clinical evaluation that the NA of patient XP11BE are due solely to her CS and not in any degree to her XP. Therefore, the nucleotide sequence defining group B has not been shown to be involved in causing premature death of neurons.

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THE RELATIONSHIP BETWEEN NEUROLOGIC DISEASE, ACUTE SUN SENSITIVITY, AND POST-ULTRAVIOLET COLONY-FORMING ABILITY IN XERODERMA PIGMENTOSUM

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ABSTRACT Neurological abnormalities and acute sun sensitivity are two clinical abnormalities associated with xeroderma pigmentosum which are not present in every patient. When we measured the ability of 24 xeroderma pigmentosum strains to divide and form colonies after ultraviolet irradiation, we found that the strains most sensitive to the ultraviolet light were derived from patients who had an early onset of neurological abnormalities, less sensitive strains were from patients with a later onset, and the most resistant strains were from patients without neurological abnormalities. Moreover, the most sensitive strains were also from patients with histories of acute sun sensitivity. These results suggest that functionally effective DNA repair is required to prevent both premature death of human neurons and acute sun sensitivity.

INTRODUCTION

Xeroderma pigmentosum (XP) is an autosomal recessive disease in which certain DNA repair processes are defective (1). Six genetic forms are currently known: the complementation groups A, B, C, D, and E, and the XP variant form. All XP patients develop signs of chronic solar skin damage, including cutaneous malignancies, at a very early age. Some patients also experience acute sun sensitivity, and some develop neurological abnormalities because of premature death of nerve cells (1). We have studied the ultraviolet (UV) sensitivity of XP fibroblast strains *in vitro* and have identified certain correlations between the clinical status of XP patients and the UV sensitivity of their cells (2, 3).

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METHODS

Log phase fibroblasts were irradiated with 254 nm light, replated at known densities, incubated at 37° for 2 to 4 weeks, and scored for colony formation (2, 3). The post-UV colony-forming efficiency of a strain expressed as a percent of the colony-forming efficiency of that strain's unirradiated cells in the same experiment was designated the post-UV colony-forming ability (CFA).

RESULTS

Post-UV CFA was determined for 24 XP strains, and the results are summarized in Figure 1. The post-UV CFA of strain XP2RO, from a group E patient, was the highest among the XP strains tested, but was significantly lower than that of any control donor strain. Lower still were the post-UV CFAs of the two variant strains and of the six group C strains tested (shaded areas, Fig. 1). No XP-associated neurological abnormalities are present in the group E, XP variant, or group C patients from whom these cell strains were derived. In contrast, all known group D patients have numerous neurological abnormalities which are usually manifested clinically by 12 years of age; each of the seven group D cell strains tested was found to have significantly lower post-UV CFA (group D shaded area, Fig. 1) than any of the group C strains. Certain group A patients have numerous neurological abnormalities which are manifested even earlier than those of group D patients. Cell strains from five such group A patients were tested, and the post-UV CFA of each (lowermost shaded area in Fig. 1) was below that of any group D strain. Three other group A strains gave very different results: the post-UV CFA of strain XP12BE, from a 7-year-old group A patient with only two neurological abnormalities, was higher than that of the group D strains but lower than that of any group C strain. XP1LO and XP8LO are from group A patients who have no known neurological abnormalities, and both strains have post-UV CFAs significantly higher than that of XP12BE. Thus, for the XP strains tested, we found a correlation between the relative post-UV CFA of the patients' fibroblasts and the time of onset of the patients' neurological abnormalities.

The impossibility of determining accurately the total sunlight exposure of XP patients or of quantifying the severity of their chronic solar skin damage precluded detecting possible correlations between post-UV CFA and the chronic skin changes of XP. However, we did find a correlation between post-UV CFA and the presence of acute sun sensitivity,

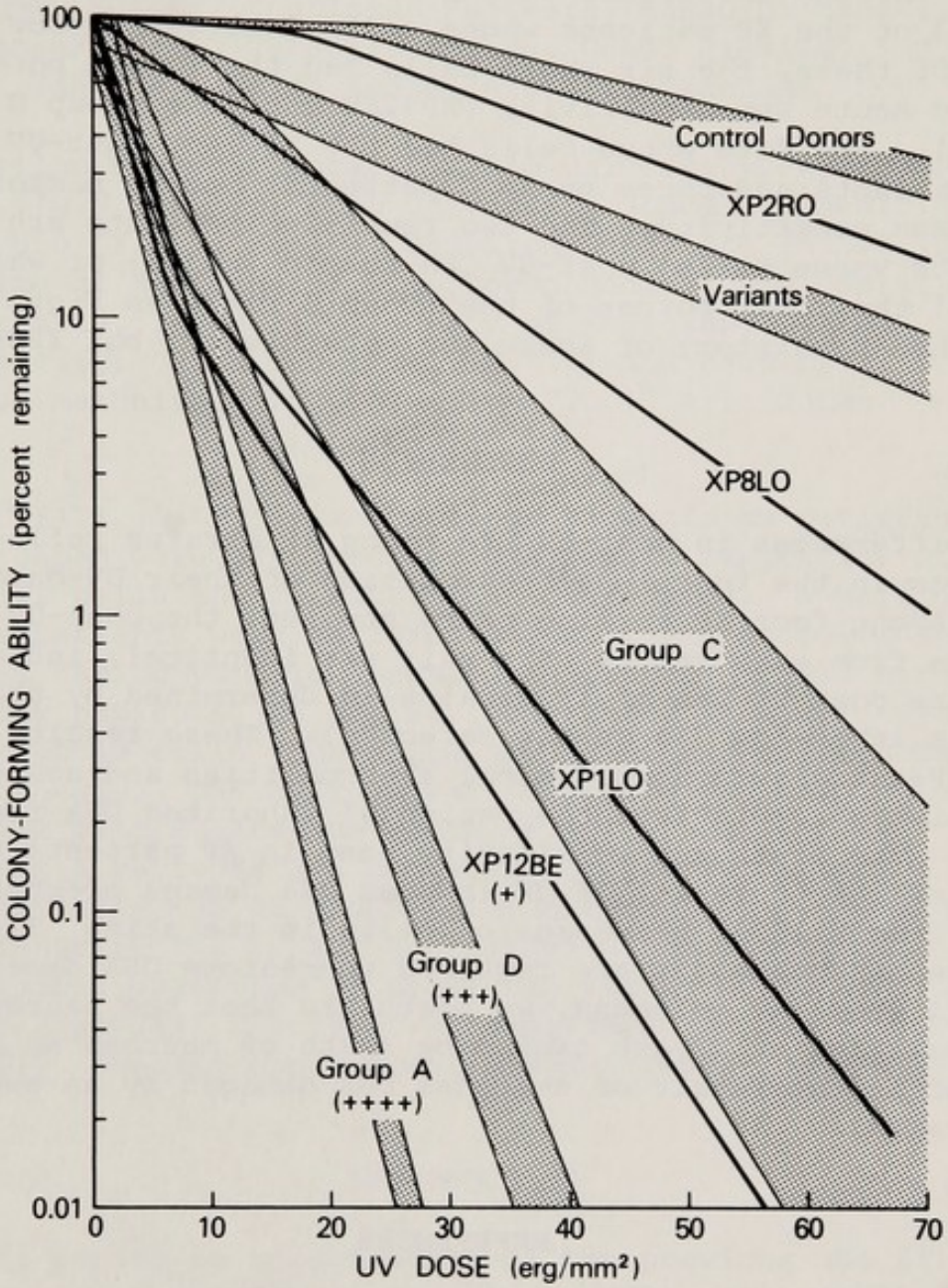

FIGURE 1. Relationship between neurological abnormalities of XP patients and the post-UV CFA of their fibroblasts. XP2RO, XP8LO, XP1LO, and XP12BE are individual strains (see text); shaded areas encompass results for 2 to 7 different strains from the indicated groups. ++++, numerous abnormalities manifested by 7 years of age; +++, numerous abnormalities manifested between 7 and 12 years of age; +, areflexia and abnormal electroencephalogram present at 7 years of age. Reproduced from reference 3.

i.e., blistering and/or severe erythema of skin after a sun exposure which does not produce such results in normal individuals.

Histories with respect to acute sun sensitivity were obtained through interviews at the National Institutes of Health with 13 of the XP patients whose cells were tested for post-UV CFA. Of these, the six whose cells had the lowest post-UV CFA all had acute sun sensitivity (XP12BE and five group D patients). The five whose cells had the highest post-UV CFA (two variants and three group C patients) had no history of acute sun sensitivity. The two remaining patients are group C patients whose cells' post-UV CFA formed (XP1BE) or was near (XP3BE) the lower border of the group C range in Fig. 1. XP1BE had no history of acute sun sensitivity, but XP3BE did.

DISCUSSION

Differences in post-UV CFA among XP strains reflect differences in the functional restoration of their UV-damaged DNA. Among four XP sibling pairs studied, the post-UV CFA of strains from each member of a pair was identical, indicating that the post-UV CFA of XP strains is determined by the patient's inherited DNA repair defect(s). These results suggest that XP-associated neurological abnormalities and acute sun sensitivity result from the patients' inherited DNA repair defects. The acute sun sensitivity seen in XP patients with the least efficient repair of UV-induced DNA damage presumably results from killing or damage of cells in the skin. Since the same repair processes are required to restore DNA damaged by certain chemical mutagens, we postulate that the neurological abnormalities of XP are caused by death of neurons as a result of inefficient repair of neuronal DNA damaged by an endogenous chemical(s).

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DNA REPAIR AND CLINICAL CHARACTERISTICS OF 96 XERODERMA PIGMENTOSUM PATIENTS IN JAPAN[1]

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ABSTRACT Ninety six xeroderma pigmentosum patients in Japan were examined for DNA repair capacities of their cells and clinical characteristics. A half of the patients were children under 10 years old, most of them having very low repair capacities. There were two older patients, 45 and 64, who showed very mild symptom but whose cells showed low amount of unscheduled DNA synthesis. One of them was identified to belong to a new complementation group, F. There were many group A patients and no group B, C, or E patients. Apparently the development of clinical symptoms were related to the repair capacities of the cells with possible exception of above two patients, although the host-cell reactivation experiment on the group F cells revealed considerably higher repair capacity.

INTRODUCTION

This report is an extention of the previous one (1) with 47 new cases of xeroderma pigmentosum (XP) patients in Japan. One patient, XP60S, was omitted from the previous report due to insufficient data. General characteristics were essentially similar to those reported, but there were a few new findings. We found a patient belonging to a new complementa-

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tion group, F. As far as XP patients in Japan are concerned, clinical characteristics seem to be related to the levels of DNA repair capacities of their cells.

RESULTS AND DISCUSSION

Age Distribution. Table 1 gives the age distribution, clinical characteristics and DNA repair as measured by unscheduled DNA synthesis (UDS) of 96 XP patients. Just a half of the patients were younger than 10 years old, when biopsy was made. The frequency of the younger patients is much higher than in other countries (Table 2), although the situation behind the survey may not be the same.

Out of these 48 young patients, 44 showed very low levels of UDS in their cells. Cells of most of the older patients had higher UDS levels. The age distribution in Table 1 suggests that generally the lower the level of repair, the earlier the symptom appear. Although it is not shown in the table, the degree of severeness of skin lesions seems to be also related to the level of repair.

TABLE I
AGE DISTRIBUTION OF CLINICAL AND REPAIR CHARACTERISTICS OF XP PATIENTS IN JAPAN

AGES	NUMBER OF PATIENTS (cancers)	MENTAL RETARDATION			UDS (% of NORMAL) (skin cancers)			
		YES	NO	UNKNOWN	5	7-15	25-60	60
0-9	48(7)	17	5	26	44(6)	1(1)	1	2
10-19	15(11)	11	4	0	12(10)	0	1(1)	2
20-29	15(11)	5	8	2	3(3)	0	3(2)	9(6)
30-39	5(3)	3	2	0	0	0	2(2)	3(1)
40	13(8)	1	12	0	0	3(1)	2(2)	8(5)
TOTAL	96(40)	37	31	28	59(19)	4(2)	9(7)	24(12)

TABLE II. AGE DISTRIBUTION OF XP PATIENTS

AGES	JAPAN (Table 1)	JAPAN(2) ('55-'60)	EUROPE(1) U.S.A.	EGYPT(1)
0-9	48	81	13	15
10-19	15	27	11	19
20-29	15	22	12	8
30-39	5	8	4	4
40	13	12	4	0
TOTAL	96	150	46	46

Host-cell Reactivation and Complementation Groups There are 3 patients belonging to 7-15 % UDS level in the age group of 40 or over, apparently not fitted to the correlation between symptoms and repair mentioned above. Two of them, aged 45 and 64, both female, had had very mild symptoms without cancers. One of them, XP23OS, showed UDS level of 10 %. XP23OS cells, however, showed considerably higher host-cell reactivation capacity than other cell lines with low levels of UDS, as shown in Table 3. This high level of host-cell reactivation capacity may reflect the total repair capacity of XP23OS causing not severe symptoms.

TABLE III

D_0 VALUES OF UV SURVIVAL CURVES OF HERPES SIMPLEX VIRUS

CELLS	COMPLE.GROUP	UDS (%)	D_0 (J/m^2)
NORMAL		100	38
XP5SE	VAR.	70	21
XP10SE		100	18
XP13OS		80	13
XP3KO	VAR.	100	12
XP23OS	F	10	11
XP5BE	D	25-55	7.5
XP9OS	A	2	5.5
XP15OS	A	2	5
XP2OS	A	2	4

By cell hybridization test, we found that XP23OS did not belong to any of existing 5 complementation groups, and should be assigned to a new group F. So far there were many group A patients, 1 D and 9 variants, but no group B,C and E have been found. One case reported as E previously was identified to be a variant by Bootsma. Table 4 gives the current status of the complementation groups and variants. It is noteworthy that group C is the most frequent in other countries.

TABLE IV GENETIC GROUP OF XP PATIENTS

Area	No. in complementation group						Variant	Total
	A	B	C	D	E	F		
JAPAN	21	0	0	1	0	1	9[a]	32
OTHER COUNTRIES[b]	15	1	19	7	2	(1)	7	52

a. by Fujiwara
b. compiled by Bootsma

Skin Cancers No patients have ever been encountered with the UDS level of 5 % or less and age of 30 or over. This may suggest that all patients belonging to this UDS level have died before 30. Skin cancers have developed in very high frequency among the patients of 10 years or older with the UDS levels of 60 % or less, with possible exceptions of two patients mentioned above (a group F patient and another in the same UDS level). More than half of the patients with UDS levels higher than 60 % have developed skin cancers after 10 years old. Since most of the children in Table 1 are with very low level of UDS, almost all of them would eventually develop skin cancers unless properly cared. Ultimately, about 90 % of XP patients in Japan have developed or are expected to develop skin cancers.

Frequency in Population Based on the frequency of the first-cousin marriage among the parents of the XP patients, which was 30 %, and the frequency of the first-cousin marriage in general population of Japan, which is assumed to be 3 % (this is changing rapidly, from approx. 6 % in 1945 to approx. 2 % now) the frequency of XP patients at birth in Japan is estimated to be 1/40000 according to Dahlberg's equation (3). This is considerably higher than the estimate made previously, 1/250000 in general population (4). One reason for this apparently high incidence could be due to the high ratio of severe cases (i.e. with very low repair capacities) which are easily identifiable in young ages. To collect 96 cases reported here, we have not tried too hard except that we organized a good cooperative research group with the support of research funds from Japanese government, consisting of dermatologists, cell biologists and geneticists.

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RESPONSE OF MAMMALIAN CELLS TO CHEMICAL DAMAGE[1]

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ABSTRACT Xeroderma pigmentosum lymphoblastoid cells derived from a group C patient (XPA3) are AP excision repair competent but are unable to carry out nucleotide excision or to remove O6 methyl guanine residues from DNA. Since uvr mutants of bacteria do remove O6 adducts, it seems likely that XPA3 is deficient in more than one enzymatic step. AP repair and nucleotide excision probably utilize separate enzymatic mechanisms for at least the first steps. Both XPA3 and an excision competent lymphoma cell line are able to replicate DNA containing benzpyrene residues. These adducts remain in the parental strand.

INTRODUCTION

Human cells respond to alterations in their DNA in at least two ways. Lesions may be excised and there are at least three variations of the excision mechanism possible depending on the lesion (1). Alternatively, lesions may be bypassed. In this paper we show that the different excision variations indicate independent pathways so that cells deficient in one are not deficient in the other. In addition we show that an excision defective xeroderma cell line is able to replicate with numerous benzpyrene adducts still present in the parental DNA. This replication is further evidence for the existence of a major non-recombinational bypass mechanism.

RESULTS and DISCUSSION

Although deficient in excision capability for UV-induced damage (Fig. 1A), a lymphoblastoid line of xeroderma pigmentosum cells, XPA3 (2) retains the ability to carry out excision repair of damage induced by methyl methanesulfonate (MMS) and methyl nitronitrosoguanidine (MNNG) (Fig. 1B,C). The repair of MNNG-induced damage by XPA3 is essentially

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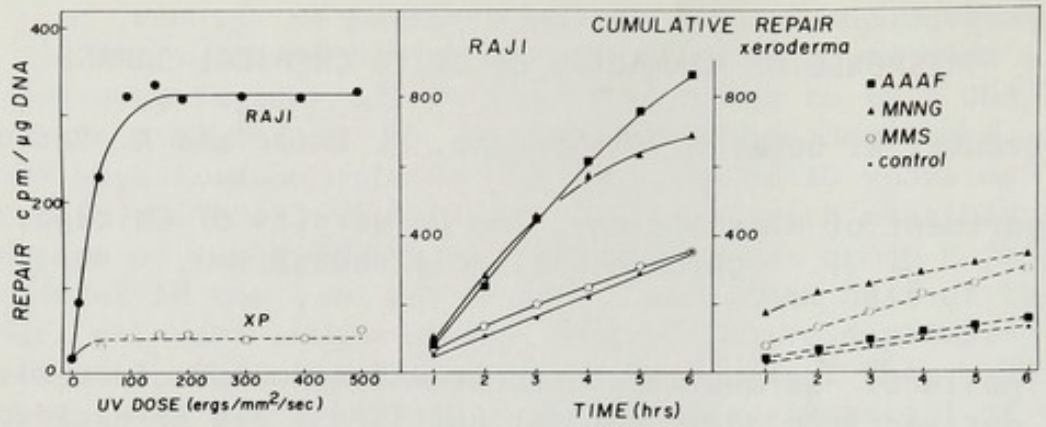

Figure 1. A. Excision repair induced by UV after one hr B.C: Cumulative repair induced by AAAF,MMS and MNNG

complete in the first hour in contrast to the kinetics observed with the nucleotide excision competent line, Raji. MMS and MNNG produce spontaneous apurinic sites (3) and the repair mode for XPA3 is likely to be AP (apurinic) repair. XPA3 cells are unable to remove O6 methyl guanine residues from DNA (Table 1), a result previously reported with XP fibroblasts (4). We used a dose of MNNG (0.5 µg/ml) which had little effect on the ability of XP cells to incorporate thymidine. Since it is not known whether O6 methyl groups are removed by nucleotide or base excision we also tested the ability of the uvr A and uvr B bacterial mutants to remove this adduct (Table 1). Although there are clearly great differences in eucaryotic and procaryotic repair mechanisms, the finding that O6 methyl excision occurs normally in uvr mutants makes it unlikely that the UV-incision enzyme is involved. Our finding is in accord with earlier results (5) in which a mutant differing from wild type by at least three genes was used.

TABLE 1

LOSS OF O6 METHYL GUANINE ADDUCTS FROM DNA

	Not Incubated			Incubated			
Line	cpm	cpm/µg	G	time	cpm	cpm/µg	G corrected for growth
Raji	156	0.5		36hrs	16	0.04	0.1
XPA3	63	0.6		36hrs	48	0.2	0.6
Escherichia coli							
wild-type	220	0.5		1 hr	0	0	0
uvr A	265	0.5		1 hr	26	0.03	0.07
urv B	267	0.3		1 hr	9	0.006	0.01
uvr AB	29	0.05		1 hr	2	-	-

Nucleotide excision proceeds with relatively few long-lived breaks present in DNA as though endonuclease were limiting (6). AP repair results in many breaks as though it were the exonuclease-polymerase-ligase sequence which limited repair. We found that the exonuclease reaction involved in in vitro AP repair was limiting and in fact that this reaction was speeded up in the presence of DNA polymerase α (Fig. 2). The patch size for nucleotide excision and AP repair is different in vivo (7) with a much smaller AP patch size as would be expected if exonuclease were limiting. Although both repair pathways involve DNA with single strand breaks, the different patch sizes as well as the different properties of the nucleases (8,9) argue that separate pathways are involved. Xeroderma pigmentosum seems to be a condition in which neither pyrimidine dimers nor O6 methyl residues are available to either nucleotide incising or (possibly) DNA glycosylases but in which AP sites are easily repaired.

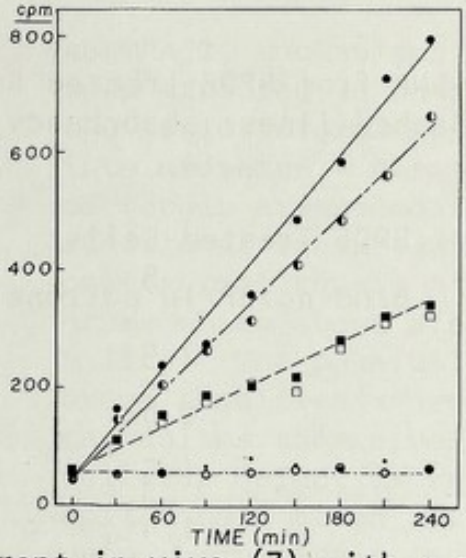

Fig. 2. Effect of DNA polymerase α on the activity of a 5'-3' exonuclease from human lymphoma cells. Top line: exonuclease + polymerase + triphosphates; Next lower: - triphosphates; Third from top: exonuclease alone; Bottom-control, no enzyme, polymerase alone. Substrate T5 DNA.

XP cells can survive and replicate in the presence of UV photoproducts even though they are excision defective (10). Replication can also occur after treatment with a chemical mutagen and most of the bound adduct remains in the parental strand. Raji and XPA3 human lymphoblastoid cells were reacted with benzpyrene diolepoxide (BPDE) at 0.05 µg/ml, a dose which does not inactivate Raji. The cells were then incubated in medium containing BrdU for a time long enough to permit replication. The DNA was then isolated by CsCl gradient centrifugation and the hybrid band wasrecentrifuged through alkaline CsCl to separate daughter and parental strands (Fig. 3A,B,C). The specific activity of the DNA was calculated from the radioactivity and absorbancy of the light, parental DNA. We found that the specific activity of the DNA in excision competent Raji cells had diminished to about 1/3 the original after 36 hours, that is at least 1/3 of the adducts remained and had been replicated (Table 2). There was almost no decrease in the specific activity of the DNA from

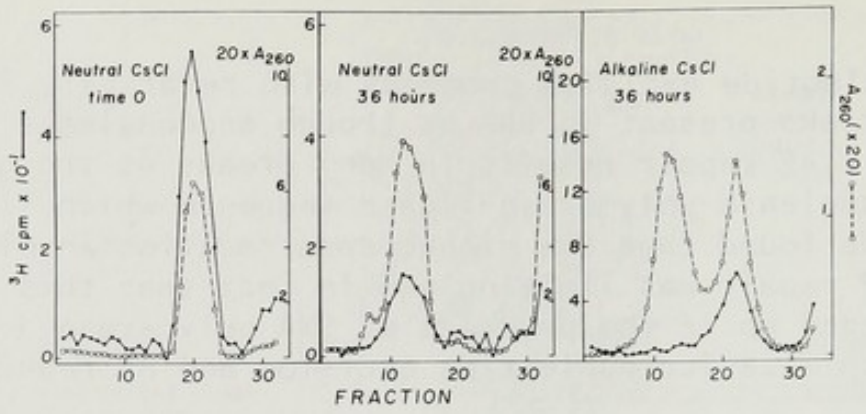

Figure 3. CsCl gradients of DNA from BPDE-treated Raji. Solid lines: radioactivity; dashed lines: absorbancy.

TABLE II

Specific Activity of DNA from BPDE Treated Cells

Cell	Time after Treatment	Binding: BP/10^8 daltons DNA
Raji	0	2.5
	36hrs	0.82
XPA3	0	1.5
	40hrs	1.4

xeroderma cells after 40 hours. The fact that almost all of the replicated adducts remain in parental DNA argues that most bypass replication occurs without recombination since a recombinational mechanism would have resulted in transfer from parental to daughter strands. It is therefore clear that even cells deficient in several excision repair steps are able to replicate past many adducts in their DNA and these adducts must therefore be present for many cell generations.

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DNA REPAIR DEFECTS AND HUMAN DISEASE[1]

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ABSTRACT Mechanisms of DNA repair have for many years been explored in nonhuman systems, and only recently has the basic information concerning them been integrated into medicine. Already, several human genetic disorders of repair are recognized; each of these presents as an additional feature chromosome instability. However, only normal repair has been demonstrable in Bloom's syndrome which shows a striking degree of chromosome instability. The possibility is presented that Bloom's syndrome signifies a new *class* of disorders in which repair mechanisms themselves are intact but in which some other cellular defect is responsible for imposition on the cell itself of an increased burden of substrates in DNA upon which repair mechanisms in turn will act.

The two manifestations of disease which have stimulated successful searches for repair defectiveness in man are sensitivity to some environmental agent and spontaneous chromosome "breakage" in cultured cells. Tumor proneness also has figured in the matter, but because dozens of disorders manifest this (1), it alone is not especially helpful. Studies of the responses made to environmentally induced DNA damage by cells from three genetic disorders—xeroderma pigmentosum, the Louis-Bar (ataxia-telangiectasia) syndrome, and Fanconi's anemia—are yielding highly interesting results (reviewed in refs. 2-4). Two of these, xeroderma and the Louis-Bar syndrome, manifest sensitivity to certain radiation, UV (ultraviolet) and γ respectively, and two show increased chromosome instability in cells grown in vitro, the Louis-Bar syndrome and Fanconi's anemia; xeroderma also shows relatively more chromosome instability than normal, but only after UV-irradiation.

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Probably additional disorders exist, including some not usually classified as genetic, with defective repair of genetic material in their pathogenesis either as a major factor or a secondary feature. Selected clinical features will be useful as clues in the search for them. Rare conditions, considered by some to be promising candidates for DNA-repair defectiveness, include Bloom's syndrome, Cockayne's syndrome, dyskeratosis congenita, incontinentia pigmenti, progeria, Rothmund's syndrome, Thomson's syndrome, Werner's syndrome, and the Wiskott-Aldrich syndrome.

Chromosome instability should remain a valuable indicator, but this sometimes is "conditional," i.e., occult or elusive until brought out by some special treatment or condition of culture; thus xeroderma cells, as just mentioned, display no excessive chromosome instability until they are treated with UV. Three rare and genetically determined human disorders, first grouped and referred to as "chromosome breakage syndromes" in 1968 (5), are Bloom's syndrome, Fanconi's anemia, and the Louis-Bar syndrome. These were not grouped because they are clinically similar or genetically related but because they all manifest two particular features—chromosome instability and predisposition to cancer. In 1972, the genetically determined chromosome breakage syndromes were compared with, and in turn grouped with, the known environmental causes of cancer—ionizing and UV irradiation, certain chemicals, and certain viruses; when so grouped and compared, they appear to constitute strong evidence that chromosome mutation is of importance in the etiology of neoplasia (6).

That human cells can manifest genetically determined defectiveness of DNA repair has also been recognized since 1968, the xeroderma studies being the first (7, 8). Because of the highly specialized and basic nature of repair investigation, the significant studies made in relation to man have, as a rule, come about through collaboration of students of repair and those interested in human genetic disease. During the DNA repair workshops held in Noordwijkerhout, The Netherlands, in May, 1976, and Alexandria, Virginia, in December, 1976, the importance of this cooperation became clear; realization of this was the source of considerable excitement in both camps. By the time of the Fifth International Congress of Human Genetics in Mexico City the same year, it was possible to describe and review a new field, that of DNA repair in relation to human disease (2). Perhaps the most interesting aspect currently of the integration into medicine of knowledge concerning the mechanisms of DNA repair is the following. The demonstration that repair defectiveness is a feature of several rare genetic syndromes that manifest chromosome instability and cancer proneness indicates also that erroneous repair it-

self is related to chromosome mutation and is of fundamental and presumably etiological importance in neoplasia (6).

Other papers in this symposium detail repair in Fanconi's anemia, the Louis-Bar syndrome, and xeroderma. The organizers invited me to present a paper on repair in "other" human disorders. The rapidity with which new knowledge in this area was accumulating was responsible for their optimism, and mine when I accepted the task. But, there is little to be said as yet about disorders of repair other than the three just named. However, one particular observation made during my search for others impresses me, and I shall discuss its possible significance instead. This is, in Bloom's syndrome defective DNA repair has *not* been demonstrable.

Unfortunately, essentially all repair studies made in Bloom's syndrome, being "negative," remain unreported. The earliest attempt was made in 1968 by John Evans and me: blood lymphocytes in short-term culture were shown to handle UV- and X-ray-induced lesions essentially normally (9). From 1969, James Regan and Richard Setlow examined extensively some Bloom's syndrome fibroblast cultures I had derived: normal excisional repair of UV-induced DNA damage was demonstrated by the bromodeoxyuridine photolysis technique and radiochromatographic analysis of the excision of UV-induced pyrimidine dimers. Rufus S. Day, III, demonstrated that these fibroblasts are capable of normal repair of UV-damaged adenovirus. Dirk Bootsma as well as the Brighton group detected no defect in fibroblasts using their various systems, which included a search for defective postreplication repair. Giannelli (10) did obtain some evidence of increased sensitivity to UV in fibroblast cultures. John Heddle and I are obtaining some evidence that Bloom's syndrome cells are somewhat more sensitive than normal to EMS, and Colin Arlett has similar evidence, but we do not know as yet just how specific this is. Bloom's syndrome cells proliferate less well in culture than do normal cells, which makes these experiments not only difficult to perform but also to interpret.

Thus, Bloom's syndrome, the prototype of the "chromosome breakage syndromes" (11), has failed to display DNA-repair defectiveness despite an extensive search. On the contrary, Bloom's syndrome cells seemingly repair very well; if a sister chromatid exchange (SCE) can be taken as cytological evidence of some repair event, these cells show themselves to be amazingly capable. Bloom's syndrome cells make many times more SCEs spontaneously than do normal cells grown in BrdU-containing medium (12). Although the search for a defect should be continued, this observation of apparently normal repair in the presence of dramatic chromosome instability may signal the existence of a previously unrecognized class of

disorders. In disorders in this class, the existence of which I wish to propose, repair would be of prime importance. However, the defect would not be in a DNA repair mechanism itself but "proximal" to the point at which DNA lesions would be recognized by the cell and repair mechanisms invoked. The defect would be different and specific in each disorder and would allow the excessive accumulation of some type substrate on which repair systems would act. The hypothesis, demonstrated schematically in Figure 1, is the following.

Normally DNA can be damaged by environmental agents (E in the figure), but repair mechanisms restore the DNA to normal and mutations (M) ordinarily are not produced. (M symbolizes mutations both visible to the cytogeneticist and invisible; essentially all suitably studied clastogens (chromosome-breaking agents) are mutagens also, and vice versa.) Increasing E abnormally, as by excessive chemical or radiation insult to the cell, II in the figure, produces more lesions in the DNA; in this case, heavy duty will be required of repair mechanisms (the heavier arrow in the figure) and a few errors (M) will be made. In cells genetically defective in some repair mechanism (dashed arrow), I in the figure, mutations occur with abnormal frequency even with a standard environmental dose of mutagens.

In the figure, C represents cellular factors which are or may be present in normal cells. By themselves they can be responsible directly or indirectly for the appearance in DNA of substrates for repair mechanisms ("lesions" in Figure I), also reparable as in the case of E-induced lesions. C, which is hypothetical, might be increased abnormally in the presence of the following: (a) genetically defective enzymes such as unwinding proteins, polymerases, and ligases which would result in lesions through sluggishness or outright erroneous action in a pathway concerned with semiconservative DNA replication; (b) defects in some cellular process unrelated directly to DNA synthesis but which could result in the accumulation, either inside the cell or in extracellular fluids, of some substance normally present in insignificant quantity that in turn is capable of producing lesions in DNA; (c) defects that result in a quantitative imbalance, either an excess or a deficiency, of some essential nutritional component of a chromosomal protein or some precursor for synthesis of the DNA molecule. In any case, C is responsible for the generation of substrates on which repair systems will be invoked to act. III in the figure depicts excessive C, the result being, just as in II and in I, an abnormally great number of M. As in II, the excessive burden of lesions would have made unusual demands on repair mechanisms—themselves normal and intact—, possibly including error-prone mechanisms.

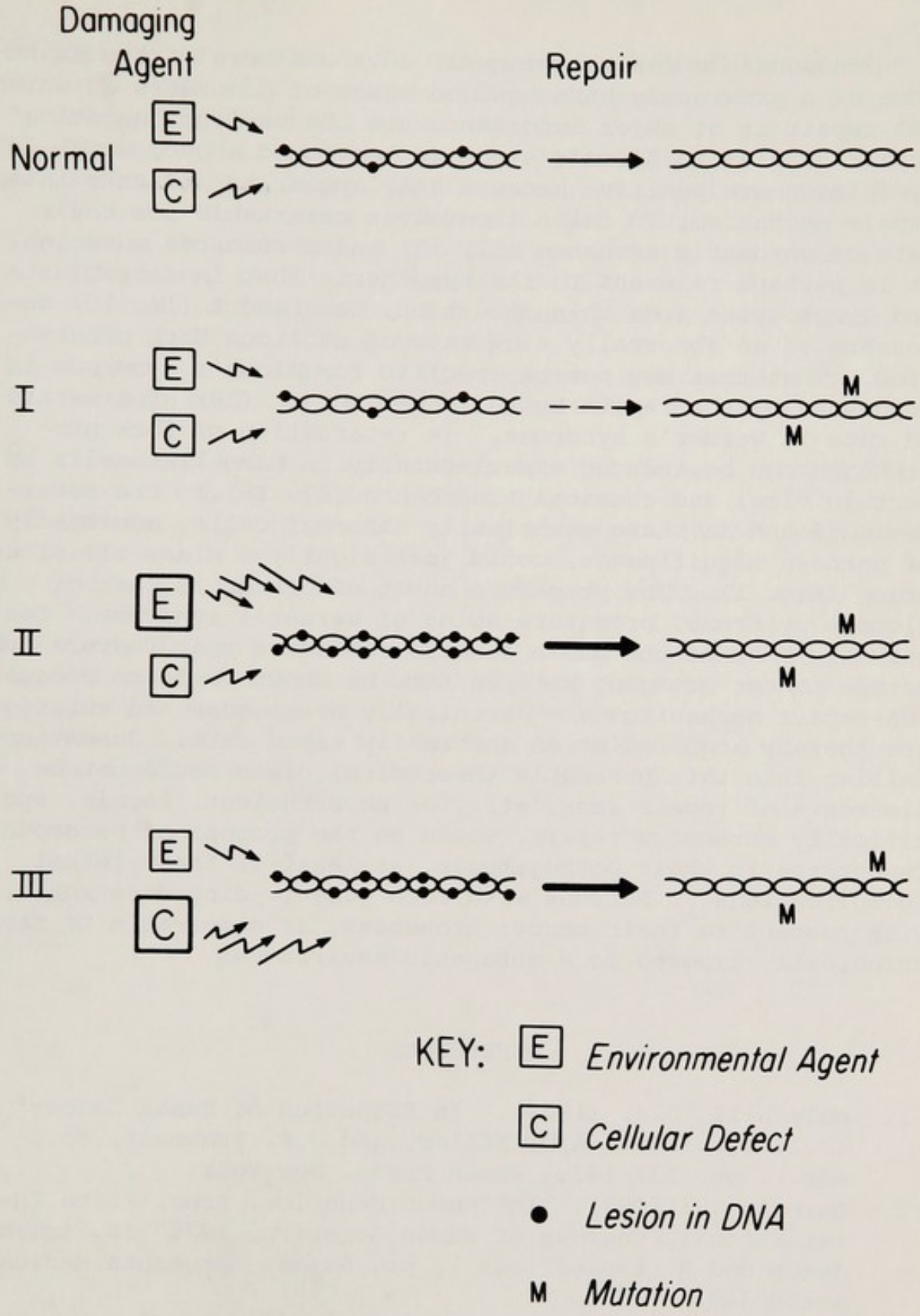

Figure 1. Mutations shown as a consequence of erroneous repair of damaged DNA in cells defective in repair mechanisms (I) or overburdened with lesions as reult of excessive damage from environmental (II) or endogenous (III) causes. (See text.)

Responsible for the proposal advanced here of the existence of a previously unrecognized class of disorders in which DNA repair is of major importance are the various "negative" repair studies in Bloom's syndrome mentioned above, which in my opinion are positive because they appear to indicate intact repair mechanisms in cells themselves remarkable for their rate of chromatid exchange (12, 13) and chromosome mutation. It is perhaps relevant to the hypothesis that in fibroblasts and lymphocytes from this condition, Hand and I (14, 15) demonstrated an abnormally slow rate of replicon fork progression. That this may not be specific for Bloom's syndrome is suggested by the report by Fujiwara **et al.** (16) of a retarded rate in Werner's syndrome. (A retardation of fork progression can be induced experimentally in mammalian cells by certain viral and chemical treatments (17, 18).) The retardation found in these genetically abnormal cells, admittedly of unknown significance, could just signify a class III situation (Fig. 1). The premature onset of cancer is part of Bloom's syndrome, premature aging of Werner's syndrome. Conceivably many of the human genetic disorders that feature premature cancer or aging will in time be shown to do so because DNA-repair mechanisms are chronically overloaded and mutations are thereby acquired at an abnormally rapid rate. Conditions falling into this presently theoretical class would not be disorders of repair *sensu stricto*; nevertheless, repair, specifically erroneous repair, would be the process of paramount importance in their pathogenesis, at least of their lethal "complications." Persons with such genetic disorders would, with respect to their cancer proneness, be comparable to those chronically exposed to a mutagenic environment.

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CELL KILLING AND MUTAGENESIS IN REPAIR-DEFECTIVE HUMAN CELLS

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INTRODUCTION: Cells from xeroderma pigmentosum (XP) patients are sensitive to 254 nm UV light when assayed in terms of cell killing (1,2) or the induction of mutants resistant to 8-aza-guanine (3). These cells are defective in excision repair (4) and/or post-replication repair (5). Other conditions such as Cockayne's (6) and Bloom's (7) syndromes and an unknown disorder shown by a sun-sensitive child [11961] (8) also confer cellular sensitivity to UV. In none of these instances, however, are there any indications of correlated defects in either excision or post-replication repair (6-9).

Cells taken from ataxia telangiectasia (AT) patients are sensitive to the lethal effects of ionizing radiation (10). Some of them have a defect in the capacity to excise gamma-ray-induced base damage (11). Sensitivity to ionizing radiation (though less than in AT cells) has also been seen in cultures from a D-deletion retinoblastoma patient (12). No defects in DNA repair have yet been identified.

RESULTS AND DISCUSSION: Cell killing. The results of a study of cross sensitivity of a variety of cell strains to an array of DNA damaging agents are summarized in the Table.

Cross sensitivity of fibroblasts from donors with different genetic disorders to a variety of DNA-damaging agents

CELL STRAIN		DNA DAMAGING AGENT					
		UV	γ	HAAF	MMS	EMS	MC
Excision defective XP	(2)	+++	-	+++	-	-	-
XP variant	(2)	+	-	-	-	-	-
11961	(1)	+++	-	-	-	+++	-
Blooms	(2)	-	-		-	+++	
Cockaynes	(2)	+++	-	-	-	+++	
Ataxia telangiectasia	(2)	-	+++	-	-		-
Fanconi's anaemia	(1)	-	++			-	+++
Retinoblastoma	(1)		++				
Progeria (Hutchinson-Gilford)	(1)		++				

+++ sensitive; - not sensitive; HAAF, N hydroxy-acetylamino fluorene; MMS, methyl methane sulphonate; EMS, ethyl methane sulphonate; MC, mitomycin C; () number of cell strains tested.

This information is based on data of the type produced in

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references 2 and 9. Some inconsistencies with published data are revealed; thus we have found that two Blooms cell strains are not sensitive to UV, whereas Giannelli et al.(7) reported sensitivity in two Blooms cell strains. This suggests that there might be heterogenity in this syndrome, as has already been observed in XP (2). Heterogeneity in sensitivity towards mitomycin C and methyl methane sulphonate in AT is also indicated (13). The sensitivity of Fanconi's anaemia cells to mitomycin C is confirmed (14). The most significant observation from our studies is the limited extent of cross sensitivity which implies that there may exist specific repair modes for each class of DNA damage.

Mutation. The results of a series of mutation experiments for 6-thioguanine resistance with UV and γ-irradiation and utilizing the techniques described by Cox and Masson (15) are summarised in figures I and II. The hypermutability of XP

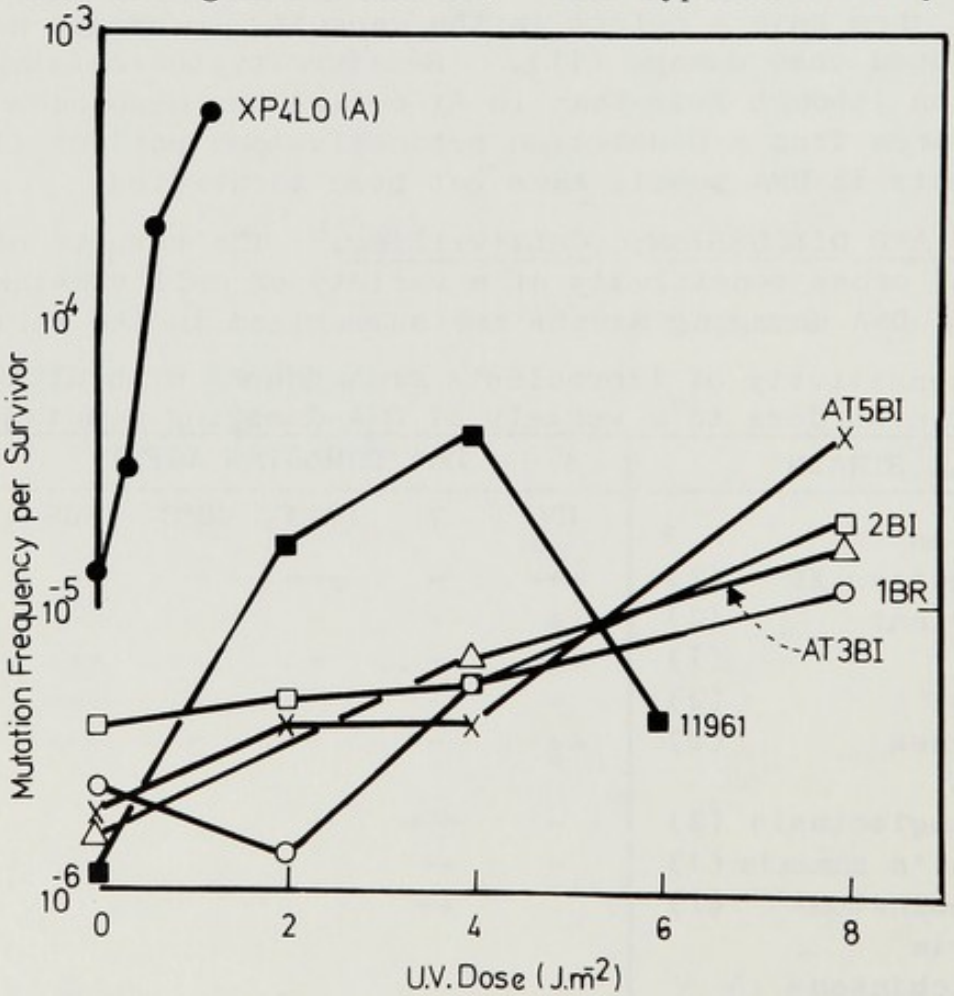

Fig.1. Thioguanine-resistant mutation frequencies in various cell strains after UV treatment. The mutation frequencies allow for expression and at these times full survival has been restored. Initial survival levels may be estimated from the D_{37} values given against the relevant strains (Jm^2). Single experiments with: XP4LO(0·25), AT5BI(5·0), AT3BI(5·0) and 1BR(5·0) normal, duplicate experiments with 11961 (0·95) a sun-sensitive individual and 2BI(5·0) normal.

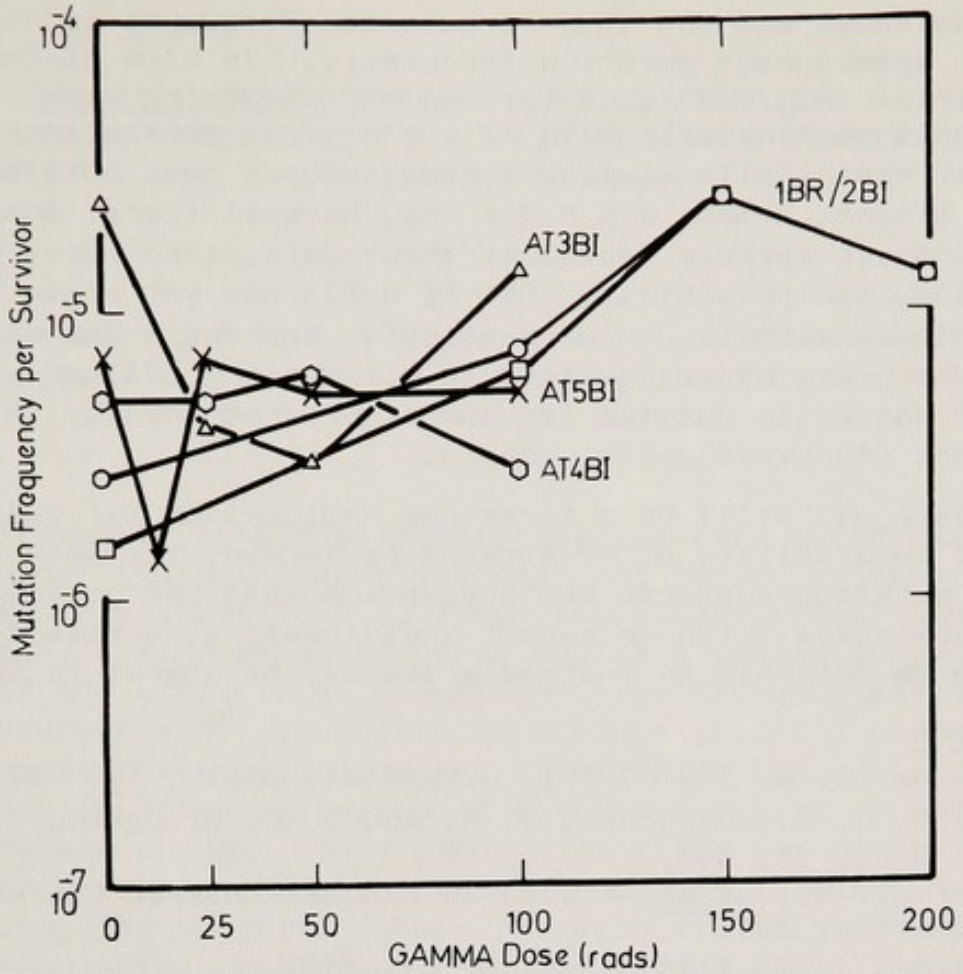

Fig. II. Thioguanine resistant mutation frequencies in various cell strains after γ-ray irradiation. The mutation frequencies allow for expression of mutants and at these times full survival has been restored in the treated samples. Initial survival levels may be estimated from the D_0 value given against the relevant strains (rads). Single experiments with 2BI (124), normal, and AT4BI(50), duplicate experiments with 1BR(160), normal, AT3BI(45) and AT5BI(43). The ataxia cell strain AT3BI is defective in the repair of γ-ray induced base damage, AT4 and AT5BI have no defect in this repair process (11).

cells (3) towards UV is confirmed with XP4LO from complementation group A. A smaller but consistent, increased mutability is also seen in cells from the sun-sensitive individual 11961 (8). Mutation frequencies induced by UV in two cell strains from normal individuals and two AT cell strains could not be distinguished.

γ-irradiation caused a 10-fold increase in mutation frequency in cells from two normal individuals over the dose range 0 - 150 r. The AT cell strains are clearly not hypermutable with γ-rays, a result which is in marked contrast to the analogous UV experiments with the hypermutable (3) XP cells. They may well be hypomutable, thus the data of Fig. II contains the results of 14 different experimental points and in only

three instances was the induced mutation frequency greater than the spontaneous mutation frequency. In view of the difficulties involved in performing and analyzing such experiments particularly with AT cells it is reassuring to note that essentially similar γ results have been obtained by Drs. Jo Simons, Leiden and Roger Cox, Harwell (pers. comm.) although these workers feel that their data, like ours, do not exclude the possibility that AT cells may yet prove to be as mutable as normals. Interestingly, Fanconi's anaemia cells, which are hypersensitive to mitomycin C (14) are reported not to be mutated (to ouabain resistance) by this same agent (Buchwald, pers. comm.).

While there may still be a technical explanation for the apparent immutability of AT strains by γ-rays, the lack of induced mutation supports the assumption that the presumptive repair processes which in normal individuals give rise to mutation in response to γ-irradiation may be absent in AT cells.

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ATAXIA TELANGIECTASIA: A MODEL INHERITED DISEASE LINKING DEFICIENT DNA REPAIR WITH RADIOSENSITIVITY AND CANCER PRONENESS

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ABSTRACT Ataxia telangiectasia, a rare hereditary disorder featuring neurovascular and immune abnormalities and predisposition to lymphoreticular cancer, exhibits many traits expected of an ionizing radiation analogue of the ultraviolet light-sensitive disease, xeroderma pigmentosum. These traits include elevated radiosensitivity extending from the clinical to the cellular level and *in vitro* hallmarks of deficient repair of radiogenic damage to DNA. This paper summarizes the present state of knowledge, draws attention to gaps in our understanding, and suggests future research directions. Speculation on the pathogenesis of the disorder implies a key role for DNA repair in embryonic development.

INTRODUCTION

In view of the increasing awareness of environmental causes of human cancer (1), it is appropriate to determine the extent to which host factors interact with the environment in cancer induction. Combined clinical and laboratory studies of certain rare genetic disorders provide an opportunity to clarify the contribution of heritable factors. Of key importance is a group of simple autosomal recessive diseases best exemplified by xeroderma pigmentosum (XP) (see Bootsma, this volume) and ataxia telangiectasia (AT). These two syndromes share certain features, including predisposition to cancer, clinical and cellular hypersensitivity to certain environmental carcinogens, and defective capacity to repair damage induced in cellular DNA by the same carcinogens (2). Continued studies on these and related diseases (see Sasaki and German, this volume) promise to shed new light on how environmentally induced damage in DNA may lead to neoplastic transformation in normal individuals.

The subject of this review is ataxia telangiectasia. Current interest in this complex, multisystem disorder largely

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centers around the unprecedented convergence of three uncommon traits: tendency to develop lymphoproliferative neoplasms, unusual sensitivity to conventional modes of radiotherapy, and *in vitro* manifestations of deficient repair of radiogenic DNA damage. The aim of this article is to summarize and evaluate recent findings, to identify gaps in our knowledge, and finally to speculate on the relation between clinical and laboratory observations and its relevancy to theories of environmental carcinogenesis.

RESULTS

Clinical Picture. The disorder is characterized by the striking association of neurological, oculocutaneous, and immunological abnormalities combined with increased susceptibility to sinopulmonary infections and lymphoid malignancies (see refs. 2 and 3 for clinical details). The first clinical sign of the syndrome is usually an unsteady gait in infancy, followed by the appearance of telangiectatic vessels initially in the bulbar conjunctivae and later in sunlight-exposed and friction areas of the skin. The gait disturbance typically progresses to severe, incapacitating ataxia by the second decade. Atrophy of the Purkinje and granular cell layers of the cerebellar cortex is the most prominent neuropathological change. The central nervous system is, however, free of telangiectasia. Death usually occurs in adolescence from respiratory insufficiency and/or lymphoproliferative neoplasia. Decreased resistance to infection is associated with an immunodeficient state, sometimes involving both the cell-mediated system (as reflected by abnormal delayed-hypersensitivity responses and poor homograft rejection) and the humoral system (as manifested by reduced or absent levels of immunoglobulins IgA and/or IgE). Whereas the neurovascular changes are constant features, the immune deficiencies vary considerably from one patient to another. An absent or rudimentary thymus is a common feature; this abnormality, besides explaining the immune defects, may also account for the stunting of somatic growth. Other clinical hallmarks of AT include: hepatic dysfunction (resulting in elevated levels of α-fetoprotein), abnormal movements of the eyes and limbs, poor or absent deep reflexes, and gonadal sterility. The absence of mental deficiency in childhood is consistent with a normal cerebrum. Progeric changes in the hair and skin, such as café-au-lait spots, are seen in patients who survive into adulthood.

Recently, still another seemingly unrelated trait has been uncovered, namely, a severe (and in some cases ultimately fatal) response to radiotherapy applied by conventional modes and in standard dosage (4). This clinical observation has

stimulated radiobiological studies of cultured fibroblasts from AT donors which have revealed deficiencies in the repair of DNA damaged by ionizing radiation.

Risk of Cancer. The incidence of cancer in affected individuals is approximately 10% (3,5); by comparison, only about 1 in 12,000 persons in the total population develop cancer before the age of 20 (6). The cancer distribution by histologic type in AT patients is: lymphoreticular, including lymphomas of the Hodgkin's or non-Hodgkin's type, 60%; leukemic, acute and chronic lymphatic types, 23%; epithelial, 13%; nervous system, 3%; and mesenchymal, 1% (5). This spectrum mimics that seen in patients with other immunodeficiency diseases but differs strikingly both from that found in the overall pediatric population and from that seen in excessively irradiated populations. Lymphomas are in the minority relative to central nervous and leukemic types in children in general (2). Acute radiation characteristically induces both acute and chronic myeloid leukemias (7); these two are conspicuous in AT patients by their absence. These and other considerations indicate that the propensity to develop cancer stems at least in part from the immunodeficient state.

Etiology. The basic cause of this autosomal recessive syndrome is unknown. Its prevalence is estimated at 24 per million children (3). The disease is widely distributed, both ethnically and geographically; Moroccan Jews in Israel are especially at high risk (8). The rate of consanguineous matings among the parents of affected persons does not appear to be higher than in the general population (3). There are clinical indications that the disease is genetically heterogeneous (9). Proneness to infection varies greatly among AT patients; however, within any one family, the severity of the sinopulmonary ailments is similar among AT siblings. Also, AT females may be distinguished on the basis of the presence or absence of ovarian dysfunction.

There are scattered reports indicating that some, but not all, AT heterozygotes manifest certain hallmarks of the disease; these include various immune defects (e.g., reduced levels of IgA), oculocutaneous telangiectases, and proneness to malignancy (3,10). Thus, it appears that mutant AT alleles may be transmitted in an incompletely recessive (partially dominant) manner.

Cytogenetic Picture. Although the modal karyotype is usually normal, elevated chromosome instability (either spontaneous or after treatment) is a characteristic trait of AT cells in culture, i.e., peripheral blood lymphocytes and dermal fibroblasts. This tendency to undergo chromosome change

prompted German (11) to classify the disease as one of the "chromosome breakage syndromes". (The others are Bloom's syndrome, Fanconi's anemia and XP.) AT cells, relative to normal controls, tend to exhibit increased levels of numerous types of gross chromosome aberrations, including gaps and breaks, dicentrics and fragments, and, in particular, non-random rearrangements (12-16). The most common type of rearrangement involves the translocation of the long arm of chromosome 14, especially at band 14q12, to terminal regions of the second chromosome 14 or, in some cases, chromosome 6, 7 or X. Of particular significance is the frequent emergence of mutant clones of lymphocytes possessing marker chromosomes arising from such rearrangements. In time, these clones of mutant karyotypes may constitute the majority of the lymphocytic population (13). In one AT patient, a lymphocytic clone with a 14q translocation actually predated the onset of chronic lymphocytic leukemia and the subsequent leukemic cells were of the same altered karyotype (16); it is therefore tempting to speculate that the neoplastic cells were derived from a pre-existing translocation clone.

Lymphocytes from AT patients present chromosome damage at levels in excess of normal upon exposure to ionizing radiation *in vitro* (17). Most noteworthy is the appearance of chromatid-type aberrations, primarily triradials, in the mitosis following G_o/early G_1 irradiation; in contrast, such aberrations are rarely seen in normal lymphocytes. This suggests that the AT lymphocytes are inept in the removal of one-strand radioproducts, e.g., either single-strand breaks or base defects. The same AT lymphocytes exhibit elevated levels of chromosome-type aberrations after G_o/early G_1 irradiation and chromatid-type aberrations after G_2 irradiation, an observation consistent with defective repair of two-strand lesions, e.g., double-strand breaks or interstrand crosslinks. In short, these cytogenetic data suggest that AT lymphocytes might be deficient in the repair of diverse classes of radioproducts and might possibly possess defects in multiple repair mechanisms.

The incidence of sister chromatid exchanges is normal in AT lymphocytes, both spontaneous (18) and after treatment with X-rays or any of three chemicals: mitomycin C (MMC), ethyl methanesulfonate, and adriamycin (19). Thus, the molecular defect responsible for the AT phenotype does not influence the yield of sister chromatid exchanges, at least in peripheral blood lymphocytes.

There are scattered reports that lymphocytes and notably fibroblasts derived from parents of AT patients show an abnormally high incidence of chromosome aberrations (12,15). As in cells from homozygous recessive persons with the disease, these abnormalities largely result from rearrangements.

Therefore, in some cases, the presence of one copy of a defective AT allele can apparently give rise to chromosome fragility. However, it is not clear to what extent these cytogenetic aberrations observed *in vitro* occur *in vivo*.

Properties of Cultured AT Cells. AT fibroblast strains proliferating in culture typically display the following abnormalities: prolonged cell generation time (15,20,21), reduced plating efficiency (22 and Fig. 1), and premature senescence (21). Difficulties in establishing fibroblast cultures from some AT donors have also been noted (15).

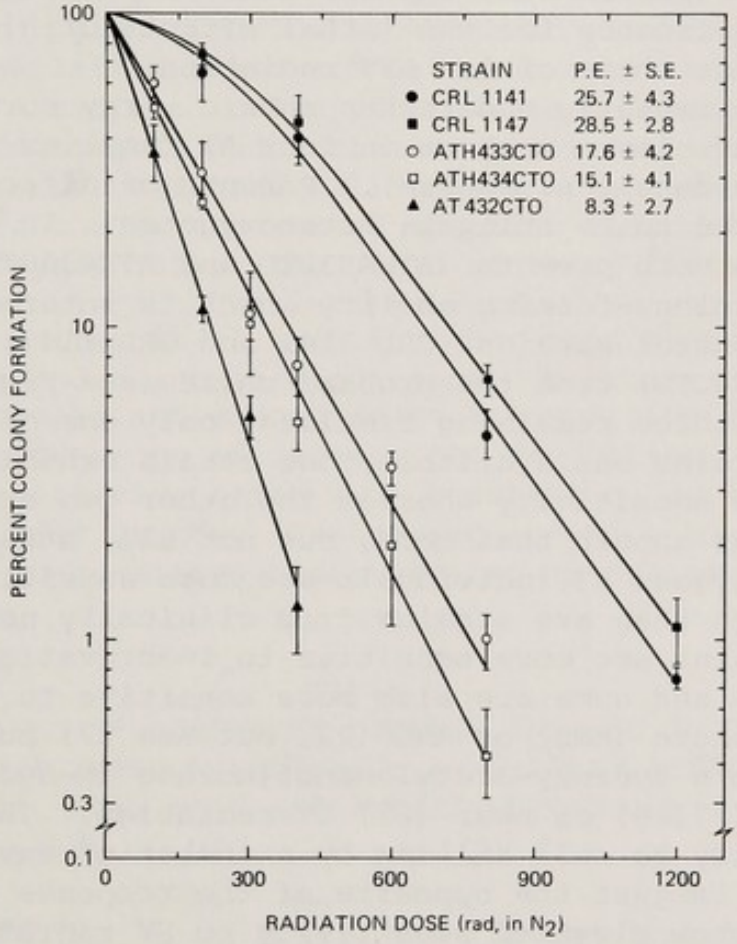

FIGURE 1. Gamma-ray survival curves of fibroblast strains from 3 members of a single AT family (proband plus both parents) and from 2 clinically normal persons. ^{60}Co γ-irradiation was administered under anoxic conditions. The symbols and error bars are the means and their standard errors of 3 or more separate experiments. P.E. ± S.E., plating efficiency ± standard error of the mean.

A phenotypic trait of AT fibroblasts that correlates with the increased radiosensitivity observed clinically is their reduced colony-forming ability, relative to that of normal cells, after acute exposure to radiations of low LET (low energy transfer) administered under either oxic or anoxic conditions (23, 24, our unpublished data, and Fig. 1). Survival curves for at least eleven genetically distinct strains of AT have now been generated and all strains are hypersensitive. In contrast to the wide variation in UV sensitivity observed among different XP strains (25), the AT strains are all about 3 times more sensitive than normal strains at the level of 10% survival. Cells from AT patients are also more sensitive to neutrons; however, the difference in sensitivity is only $\sim$1.4 (26) so that the defect in repair in AT cells is of less significance for the lethal effects of high LET radiation than for those of low LET radiation.

We have recently undertaken anoxic γ-ray survival studies on a total of eleven members in four AT families (data to be published in detail elsewhere). Parents of affected patients are considered to be obligate heterozygotes. In one family, strains from both parents (ATH433CTO and ATH434CTO) display a level of colony-forming ability which is intermediate between two control strains (CRL 1141 and CRL 1147) and the AT strain (AT432CTO) from the proband child (see Fig. 1). In each of the three remaining families, only one of the two parental strains was available; one strain exhibits a similar intermediate sensitivity whereas the other two are normal. It would thus appear that some, but not all, strains derived from heterozygous AT individuals are more sensitive to anoxic γ-irradiation than are strains from clinically normal persons.

AT strains are more sensitive to inactivation by actinomycin D (22) and some are also more sensitive to methyl methanesulfonate (MMS) or MMC (22, but see 27) but are not sensitive to N-hydroxy-acetylaminofluorene (N-hydroxy-AAF) or either far (27,28) or near (27) UV radiation. This pattern of sensitivity to cell killing by a number of environmental carcinogens is just the opposite of the response of XP cells; the latter show elevated sensitivity to UV radiation (25,29) or N-hydroxy-AAF (29) but not to ionizing radiation or MMS (27).

Transformation of AT cell strains by SV40 virus occurs at a normal rate (21); consequently, the basic defect does not affect the mechanism promoting the insertion of DNA from this oncogenic virus into the human host genome. It would be of interest to determine the influence of radiogenic damage to viral and/or host DNA on the transformation rate in AT compared to normal cells.

DNA Repair Properties of AT Fibroblasts. The discovery that AT cell strains are hypersensitive to radiation has prompted physicochemical studies into the repair of radiogenic DNA damage in such strains. Whereas AT strains are proficient in rejoining both single-strand (23,30,31) and double-strand (32) breaks, approximately one-half (seven of twelve analyzed) are deficient in carrying out DNA repair replication after anoxic γ-irradiation (33, our unpublished data).

To define the repair properties of AT strains, we have employed an enzymatic assay (34) to monitor the initial yield and subsequent metabolic fate of two classes of radioproducts in AT and normal cells. These are single-strand breaks (frank scissions plus alkali-labile bonds) and base defects (altered base and sugar moieties not giving rise directly to an interruption in the polynucleotide backbone at alkaline pH). Members of the latter class are detected as sites in DNA (extracted from irradiated cells after various incubation times) which are sensitive to endonuclease activities in a crude extract of *Micrococcus luteus* (31) or (in more recent unpublished studies) to the single-strand specific endonuclease S_1 from *Aspergillus oryzae* (35). The yield of these radioproducts is presented in Table 1. The incidence of *M. luteus* endonuclease-sensitive sites is ~1.5 fold higher than that of single-strand breaks under anoxic conditions. Whereas the yield of both strand breaks and endonuclease S_1-sensitive sites is enhanced by the presence of oxygen, the production of *M. luteus* endonuclease-sensitive sites is, if anything, decreased during irradiation under oxygen. The degree to which the two types of endonuclease-sensitive sites overlap is unknown. The

TABLE 1
YIELD OF DIFFERENT DEFECTS INDUCED IN DNA OF HUMAN FIBROBLASTS BY γ-IRRADIATION UNDER OXIC AND ANOXIC CONDITIONS

TYPE OF DEFECT	N_2		AIR	
	eV/defect*	Relative Yield¶	eV/defect*	Relative Yield¶
Single-strand breaks‖	73	1.0	33	2.2
M. luteus endonuclease-sensitive sites	50	1.46	61	1.2
Endonuclease S_1-sensitive sites	140	0.52	100	0.73

* Energy absorbed in DNA per defect from (eV/defect)·(defect/dalton rad) = 104×10^{-12}

¶ Normalized to single-strand breaks in N_2

‖ Plus alkali-labile bonds

hypersensitivity of the AT strains is probably not due to a higher level of radiogenic damage since the yield of all three types of radioproducts is the same in AT and normal strains. The presence of normal levels of superoxide dismutase and catalase in cell-free extracts of AT strains (36) is consistent with AT and normal cells sustaining equal amounts of damage by radiation-induced oxygen radical species.

In three repair replication-deficient AT strains [AT3BI, AT1BE (CRL 1312), AT81CTO] tested to date, the disappearance of *M. luteus* endonuclease-sensitive sites is protracted relative to that seen in normal strains; the rate of site loss is about 2-3 fold slower in the AT strains than in the normal strains (31; our unpublished data). Our preliminary data on the disappearance of endonuclease S_1-sensitive sites and single-strand breaks for AT3BI and CRL 1141 cells is given in Fig. 2. In keeping with studies cited earlier (23,30,31), the single-strand breaks rapidly disappear from both strains so that within 30 min only ∿20% of the original breaks remain in the DNA of either cell type. In contrast, whereas ∿90% of the endonuclease S_1-sensitive sites are gone by 30 min in the normal cells, even after 120 min ∿60% of these sites remain in the AT cells. These results suggest that certain AT strains are deficient in one or more endonucleases needed to initiate

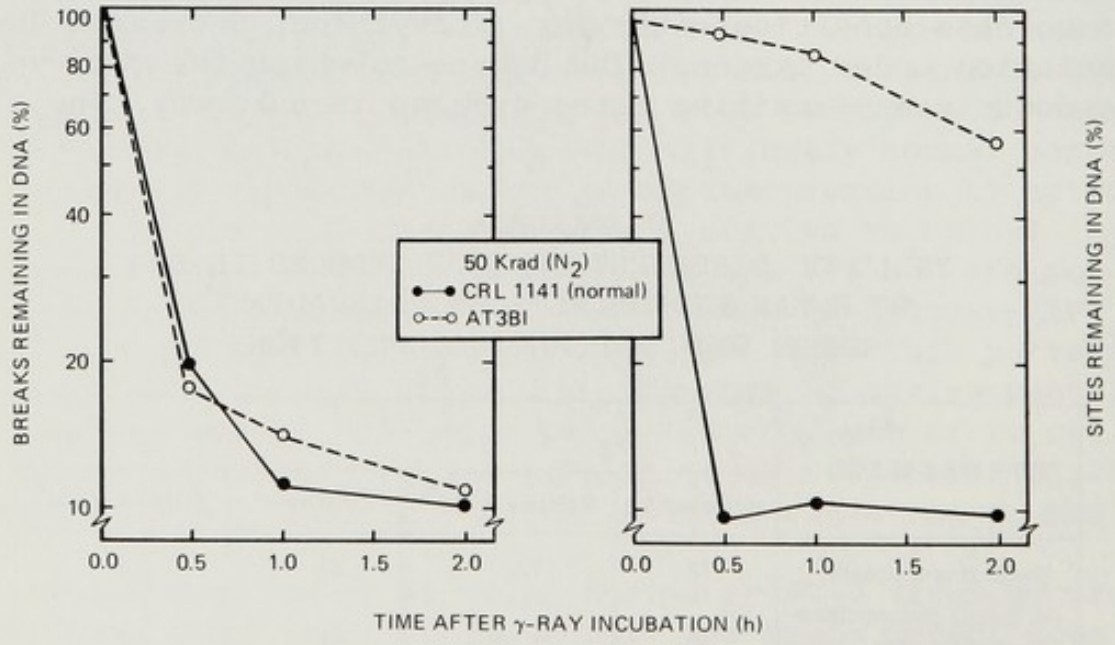

FIGURE 2. Loss of γ radioproducts from the DNA of irradiated human cells. Single-strand breaks (left panel) and endonuclease S_1-sensitive sites (right panel) were monitored by an *in vitro* enzymatic assay (31,34), using purified endonuclease S_1 of *A. oryzae* (Miles Lab. Inc.) as the lesion probe (35).

excision of γ-induced base defects; the data are also consistent with the reduced levels of repair replication observed in these strains (33). The γ lesions at sites recognized by the *M. luteus* extract and endonuclease S_1 preparations have not been defined chemically. Presumably, a fraction is a class typified by 5,6 dihydroxydihydrothymine. This class of damaged thymines is excised normally from human cell chromatin by whole cell sonicates of both repair replication-deficient and -proficient AT cell strains (37); however, certain AT strains may still be deficient in the removal of this class if, as has been found for UV-induced pyrimidine dimers and sonicates of XP variant strains (38), the conditions *in vitro* do not mimic those in living cells. Since the action of endonuclease S_1 on native DNA is believed to be confined to regions of extensive denaturation (39), AT3BI cells may be impaired in the removal of a class of γ lesions which cause gross helix distortions (e.g., DNA-protein crosslinks) and thus may possibly elicit a "long patch, UV-like" repair process (40).

The five AT strains with a normal capacity to perform repair replication after anoxic γ-irradiation have been termed AT variants (33) by analogy to the XP variants which carry out UV-induced repair replication normally (41). In fact, one AT variant strain, AT4BI, has been studied further; this strain removes *M. luteus* endonuclease-sensitive sites normally (33). Hence, the variant strains may excise γ radioproducts proficiently just as XP variants excise UV photoproducts proficciently (41). However, the analogy between the AT and XP variants is not complete; variant strains are more prevalent in AT (33) than in XP (28). Moreover, the UV sensitivity is much less pronounced for XP variants than for excision-defective XP strains (25) while all AT strains are, as noted earlier, about equally sensitive to ionizing radiation (23,24, our unpublished data).

Cell hybridization studies (33) have assigned three AT strains defective in γ-induced repair replication to two complementation groups: AT3BI and AT1BE in group A and AT2BE (CRL 1343) in group B. The variant strains constitute a third group. Complementation between AT strains provides evidence that genetic heterogeneity can exist in the disease, different mutant loci giving rise to the observed deficiencies in γ-induced repair replication. Heterogeneity is further suggested by the existence of AT strains which are proficient in repair replication and others which are deficient.

AT strains appear to cope with damage caused by far UV radiation or MMS treatment in a normal fashion. In keeping with a proficient excision-repair capability, such strains act on UV-endonuclease-sensitive sites with normal kinetics and exhibit normal levels of UV-induced repair replication (31,33). AT strains also appear efficient in carrying out postreplication

repair after UV exposure, since they show no defect in converting DNA synthesized on UV-damaged template from initially low to high molecular weight (28). MMS treatment induces normal amounts of repair replication in AT strains, suggesting that such strains can readily repair alkylation products formed in the DNA by this chemical carcinogen (our unpublished data).

The relative capacities of AT heterozygous strains to perform γ-induced repair replication mimics that found for cell survival; those strains exhibiting a moderate sensitivity to killing also perform moderately reduced levels of repair replication, and those strains exhibiting normal survival carry out repair replication normally (our unpublished data). This parallelism between colony-forming and repair replication abilities suggests that the capacity to carry out repair replication reflects functional repair and not simply some pathogenic response to ionizing radiation.

DISCUSSION

Possible Role of Repair Deficiency in Pathogenesis of AT. The connection between the clinical and laboratory observations in AT is not obvious. Several theories have been advanced to explain the mode of development of this complicated disease (2,33,42). Abnormal tissue differentiation during early embryogenesis could account for many clinical features including the immune, hepatic and gonadal dysfunctions (43); thus, efficient repair of DNA damage may be particularly important during the first trimester of fetal development (33). The progressive deterioration of the cerebellum may stem from neuronal death caused by inefficient repair of damaged DNA. Neurons are not replaced after about two years of age and fully functional repair mechanisms may be required to maintain the integrity of the central nervous system (2). The predominance of lymphoproliferative neoplasms in AT patients correlates with the immunodeficient state. Deficient DNA repair may be causally linked by permitting, for example, a disproportionate elevation in the number of potentially cancerous cells in the cellular and humoral immune systems.

Gaps in our Knowledge: Future Research. AT has many of the properties expected of an ionizing radiation analogue of the UV-sensitive disease, XP. However, our understanding of the primary defect in AT is in its infancy. The basis of the increased sensitivity of AT variants to radiation remains unexplained and, even in the AT strains which are deficient in repair replication, there is no clear evidence whether unexcised base defects are the lesions responsible for their hypersensitivity to radiation. The similarities in pattern of

chromosome aberrations and in degree of radiosensitivity among all AT strains is consistent with one or more deficiencies in a common repair pathway. Lehmann (44) has speculated that inefficient repair of a minor undetectable fraction of the strand breaks, rather than of a class of base defects, is responsible for the increased radiosensitivity of AT strains.

It has been estimated that ∿1% of the general population are AT heterozygotes, i.e., carry one defective copy of an AT gene (10); if, as seems probable from the complementation studies, a mutation in any one of several different genes gives rise to the AT phenotype, then this estimate would be even higher. Thus, the observation that certain AT heterozygous strains are hypersensitive to γ-rays is of public health concern, particularly since blood relatives of AT patients are known to be prone to malignancy (10). [It should be stressed, however, that the harmful agent(s) in the natural habitat is unidentified and may be a chemical carcinogen(s), rather than natural background radiation; the critical lesions may also arise spontaneously.] It is conceivable then that defective copies of an AT gene may contribute more to the cancer burden than thought originally.

Although numerous avenues of future research deserve attention, six predominate: (1) Measure the mutability of AT strains by ionizing radiation to determine whether AT lends itself to the somatic mutation theory of carcinogenesis (45); (2) Resolve whether deficient DNA repair is the basic biochemical defect in AT; (3) Determine the sensitivity of AT cells to such chemical carcinogens as N-methyl-N'-nitro-N-nitrosoguanidine and benzo(α)pyrene; (4) Develop new technology to follow the fate of chemically defined radioproducts; (5) Perform complementation analysis on more AT strains to simplify the task of defining repair deficiencies in AT; and (6) Search for animal models of AT; such models would facilitate indirect assessment of the interaction of genetic and environmental factors in the pathogenesis of the disease. Success in carrying out these projects should increase our understanding of AT and also provide important information on the contribution of environmentally induced DNA damage and its incomplete repair to the ill-health of man.

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ATAXIA TELANGIECTASIA : CHARACTERIZATION OF HETEROZYGOTES[1]

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ABSTRACT Determination of ionizing radiation induced DNA repair in phytohemagglutinin (PHA) stimulated lymphocytes distinguishes ataxia telangiectasia (AT) homozygotes and controls but does not provide a means of detecting heterozygotes. The increased radiosensitivity demonstrated in AT fibroblasts is also apparent in Epstein Barr Virus (EBV) transformed lymphoblasts. At an ionizing radiation dose of 400 rads viability curves for AT homozygotes, AT heterozygotes and controls have a trimodal distribution as monitored by dye exclusion. Discrimination is also demonstrable between dose response curves of cells cloned in agar.

INTRODUCTION

A number of human genetic disorders have been described which show increased sensitivity to radiation and defective repair of DNA damage (1-3). These disorders are also characterized by immune disturbance and CNS abnormalities (1,2). It is suggested that these abnormalities may be correlated to the defective DNA repair observed in these syndromes (4,5).

Fibroblasts from patients with AT, an autosomal recessive disorder with increased frequency of chromosomal breakage, show an increased sensitivity to ionizing radiation (6), and in some cases defective DNA repair (5). However no such biochemical abnormalities have been demonstrated in AT heterozygotes although an increased propensity to cancer is observed in these individuals (7).

We have attempted to provide a rapid assay to measure deficits in DNA repair in AT cells after exposure to ionizing radiation, and extend this to heterozygotes as a means of detection. PHA stimulated lymphocytes and EBV transformed lymphoblasts have been used in this study. The ready availability of lymphocytes and the large increase in repair capacity observed in PHA stimulated lymphocytes relative to unstimulated cells (8) provide a rapid and useful assay.

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RESULTS AND DISCUSSION

Table 1 provides data on DNA replication and repair in stimulated lymphocytes 96 hr after addition of PHA.

TABLE 1
DNA REPLICATION AND REPAIR IN PHA STIMULATED LYMPHOCYTES[a]

Lymphocyte	DNA Repair cpm/10^6 cells [$\times 10^{-3}$]	DNA Replication cpm/10^6 cells [$\times 10^{-5}$]	DNA Repair/DNA Replication (normalized)
Control	6.1 ± 0.8	4.43 ± 2.66	0.014
AT Heterozygote	6.5 ± 2.4	5.26 ± 2.98	0.015
AT Homozygote	1.0 ± 0.2	2.91 ± 0.50	0.004

[a]Cells were irradiated (40 Krads) and labelled as previously described (8). DNA repair is calculated as the difference between [^{3}H] thymidine incorporation (in the presence of hydroxyurea) in irradiated cells and in unirradiated cells. Incorporation was normalized to 500,000 cpm for DNA replication.

The results show that the level of DNA repair is approximately 5-fold lower in AT homozygotes than controls. Heterozygotes however are in the same range as controls. DNA repair is induced coincidentally with DNA replication in PHA stimulated lymphocytes (8), and a constant ratio of repair to replication is observed on day 3 and day 4 after addition of PHA. Since some variation occurs in the timing of the peak of synthesis after PHA it is appropriate to express the results as a ratio of repair to replication. Using this ratio, controls and heterozygotes were indistinguishable whereas AT homozygotes had a 4-fold reduction. Recent results (9) using inhibition of DNA replication as a measure of ionizing radiation induced repair in lymphocytes have also demonstrated a decreased level of DNA repair in AT homozygotes. While this assay system described here distinguishes homozygotes and controls it is unable to distinguish between heterozygotes and controls.

This question was pursued using EBV transformed lymphoblasts. These cells have the advantage over lymphocytes that they can be maintained in culture indefinitely.

The sensitivity of AT homozygote, heterozygote and control lymphoblasts to ionizing radiation was determined by

measuring colony forming ability in agar (10). The results are expressed as D_o values (dose required to reduce survival to 37%) in Table II.

TABLE II

Cell	D_o (rads ± S.D)
Control (2)[a]	98.3 ± 11.7
AT Heterozygote (3)	74.5 ± 6.7
AT Homozygote (2)	30.5 ± 0.7

[a]Number of individuals tested

In these experiments the D_o value for AT homozygotes was considerably lower than in controls. A difference is also observed between heterozygotes and controls but because of the small sample size in both cases the significance of these data can be established only by the inclusion of further cell lines. These differences are more evident when sensitivity to ionizing radiation is assayed by measuring cell viability, using trypan blue exclusion from cells.

Viability was determined at 48 hr and 96 hr after exposure of cells to ionizing radiation (400 rads). The results of these viability studies appear in Table III.

TABLE III

Cell	Cell viability (Nt/No)	
	48 hr	96 hr
Control[a]	1.32 ± 0.1	1.80 ± 0.4
AT Heterozygote	0.88 ± 0.04	0.98 ± 0.06
AT Homozygote	0.59 ± 0.1	0.36 ± 0.08

[a]Viability studies were carried out on 8 controls, 3 heterozygotes and 5 homozygotes.

After 48 hr control cells showed an increase in viable cell number and at 96 hr this increased to a value approximately double that of the initial cell numbers. Homozygotes were reduced to 0.6 of the initial cell number 48 hr after

irradiation to 0.35 at 96 hr. After an initial decrease heterozygote cell numbers remained largely unchanged up to 96 hr.

While this method does not provide a quantitative estimation of cell survival, it clearly reflects the pattern obtained using agar cloning and provides a more rapid and more sensitive means of distinguishing controls and AT heterozygotes.

It is not clear whether this decreased viability in heterozygotes is due to an anomaly of DNA repair or replication.

ACKNOWLEDGMENTS

We wish to thank Karen Baker for technical assistance, and Denis Moss for EBV transformations.

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REPAIR DEFICIENCY IN N-METHYL-N'-NITRO-N-NITROSOGUANIDINE TREATED ATAXIA TELANGIECTASIA FIBROBLASTS

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ABSTRACT After treatment with N-methyl-N'-nitro-N-nitrosoguanidine, three Ataxia telangiectasia cell lines exhibited 21-34% of the DNA repair synthesis shown by normal human cell lines as estimated using the benzoylated naphthoylated DEAE cellulose method. Two other Ataxia lines were normal in this assay. Furthermore, all Ataxia lines tested had less than normal colony forming ability after nitrosoguanidine treatment. By comparison, all Ataxia lines had normal levels of DNA repair synthesis after treatment with methyl methanesulfonate or ultraviolet light.

INTRODUCTION

Fibroblasts derived from patients with the autosomal recessive disease Ataxia telangiectasia (AT) are unusually sensitive to ionizing radiation, exhibiting increased chromosomal aberrations, depressed levels of DNA repair synthesis, and enhanced cell lethality (1-8). Paterson and coworkers have reported recent cell fusion studies indicating genetic heterogeneity in AT, thus allowing the assignment of AT strains to two complementation groups showing gamma-ray induced repair deficiency and another group with normal levels of repair replication (8). AT cell lines appear normal in their response to ultraviolet light (UV) (6-9); however, not enough data has been reported to give a clear pattern of their response to chemical carcinogens (6,10). In this investigation the effects of UV, methyl methanesulfonate (MMS), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on DNA repair in some AT cell lines are reported.

RESULTS AND DISCUSSION

The benzoylated naphthoylated DEAE cellulose (BND-cellose) method for the rapid quantitative estimation of

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excision repair and the rigorous separation of repair synthesis from semiconservative replicative synthesis has previously been well documented (11-13). The specific repair activity induced by any treatment can be calculated from the radioactivity and absorbance at 260 nm of the 1.0 M NaCl eluate of a BND-cellulose column. To measure repair synthesis, log phase fibroblast cultures grown in Dulbecco's modified Eagle medium (GIBCO) were preincubated in 10 mM hydroxyurea (Hu) for 30 minutes followed by: 1) 60 minutes incubation in medium with either MMS or MNNG, 10 mM Hu, and 3-H thymidine (10 uCi/ml, 18 Ci/mM, Schwarz/Mann); or 2) washing with PBS, UV irradiation, and incubation for 60 minutes with Hu and 3-H thymidine. Cell lysates from normal and AT cell lines were eluted from BND-cellulose as previously described (11-13). Repair synthesis was measured at 11 drug concentrations and 12 different UV doses. At least 6 independent experiments were performed for each cell line with each drug or UV. The maximum levels of repair synthesis measured for the different cell lines studied are given in Table 1. UV-induced repair synthesis is approximately equal for all 10 lines tested. Similarly, approximately equal

TABLE 1 [a]

MAXIMUM REPAIR SYNTHESIS (CPM/ug DNA)

Cell Lines	MMS	UV	MNNG
Normal			
CRL 1295 (KD)	48.5	305.1	109.5
CRL 1220	55.6	309.1	106.4
CRL 1187	57.2	260.0	106.8
CRL 1224	62.2	287.1	115.4
WI 38	48.4	331.8	124.1
Ataxia telangiectasia			
CRL 1312	47.3	294.3	24.0
CRL 1343	69.9	300.9	38.0
AT3BI	47.1	289.3	35.9
AT5BI	45.1	331.2	89.8
CRL 1347	55.5	339.5	115.4

[a] Data in table are from concentrations of MMS: 200-260 ug/ml; MNNG: 30-40 ug/ml; and UV doses: 10-20 J/m2. AT3BI and AT5BI were provided by C. Arlett, and the other 8 lines were obtained from the American Type Culture Collection, Rockville, Maryland.

amounts of repair synthesis are induced by MMS in all 10 lines. However, MNNG-induced repair clearly shows a different pattern. The 5 normal cell lines and two AT lines (CRL 1347 and AT5BI) show similar levels of MNNG-induced repair synthesis. Three AT lines (CRL 1312, CRL 1343, and AT3BI) exhibit significantly less repair than do normal lines. Paterson has reported diminished gamma-ray-induced repair replication in these same 3 AT lines and normal repair in CRL 1347 and AT5BI (8).

In order to determine whether this diminished ability to repair MNNG-induced damage is reflected on the biological level the effect of MNNG treatment on colony formation was examined for some of the cell lines. Log phase cultures growing in Nutrient mixture F12 (GIBCO) were treated in medium with various MNNG concentrations for 60 minutes, washed with PBS, and incubated for 2 weeks. Figure 1 shows colony formation after MNNG treatment. Both

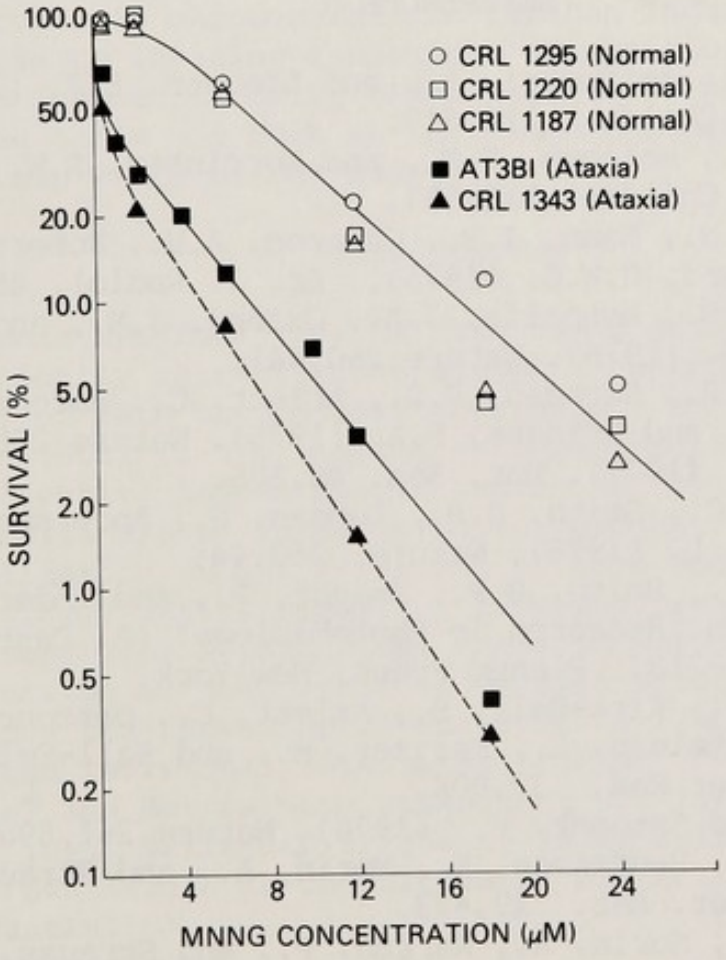

Figure 1 Effect of MNNG on survival of human fibroblasts as measured by colony formation. Cultures were incubated with MNNG in medium for 60 minutes.

AT cell lines (AT3BI and CRL 1343) show significantly enhanced sensitivity to MNNG treatment. Post MNNG colony forming experiments done with the two AT lines showing normal levels of repair synthesis (CRL 1347 and AT5BI also reveal diminished cell survival after MNNG treatment (data not shown. Thus, AT cell lines showing either reduced or approximately normal levels of MNNG-induced DNA repair synthesis, show decreased survival after MNNG treatment. Paterson (8) has reported approximately normal levels of gamma-ray-induced repair replication in an AT cell line shown by Taylor and co-workers (5) to have greatly decreased survival after radiation treatment. Thus, MNNG appears to be radiomimetic in human cells and the pattern of repair and cell survival observed after MNNG treatment parallels the findings of Paterson for AT cell lines treated with ionizing radiation.

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AN INVESTIGATION OF DNA REPAIR POTENTIAL IN BLOOM'S SYNDROME[1]

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ABSTRACT Bloom's syndrome is characterized cytogenetically by increased frequencies of chromosomal aberrations and sister chromatid exchanges. We have earlier suggested that these increases are due to the endogenous production of an agent(s) capable of damaging DNA, and not due to a DNA repair deficiency. In an examination of DNA repair potential, Bloom's syndrome cells exhibited an increase in UV-induced unscheduled DNA synthesis and a decrease in chromosomal aberrations induced by a G_2 exposure to ionizing radiation. These findings suggest that instead of being DNA repair deficient, Bloom's syndrome cells may have an increased capability of handling certain kinds of DNA damage.

INTRODUCTION

Bloom's syndrome (BS), a rare human genetic disorder, is cytogenetically characterized by an increased "spontaneous" frequency of both chromosomal aberrations and sister chromatid exchanges (SCE) (1-4), leading to suggestions that BS cells may be defective in DNA repair and/or DNA synthesis (5,6). Yet, BS cells appear to be normally competent in excision and post-replication repair (6,7) and to have normal DNA polymerase (α,β,γ) activities (8). Recently, we proposed that these cytogenetic findings may arise in part from the endogenous production of some agent(s) capable of damaging DNA (9). This theory was tested by co-cultivating fibroblast cells from an affected individual with cells from an appropriate control individual and determining subsequent SCE levels in both types of cells (9). While SCE frequency in BS cells remained unchanged, SCE levels in co-cultivated control cells increased significantly.

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Subsequently, we have attempted to characterize the repair potential of BS cells in order to further rule out a possible DNA repair defect. Two basic systems were employed: ultraviolet (UV) -light-induced unscheduled DNA synthesis (UDS) and chromosomal aberration induction by ionizing radiation during the G_2 portion of the cell cycle.

RESULTS

UV-Induced UDS In BS and Control Fibroblast Cells. UV-induced UDS was examined in fibroblast cultures of both BS and control origin, cultured as described previously (9). Fibroblast cells, attached to round glass coverslips (25 mm dia, Bellco) and allowed to become confluent, were rinsed in PBS, exposed to UV light (primarily 254 nm, 7.5 J/m^2/sec) and subsequently incubated in complete medium containing [^{3}H]-thymidine (1 μC/ml, 6 C/mM, New England Nuclear) for various times. The coverslips were fixed in Carnoy's solution, attached to microscope slides, dipped in Kodak NTB emulsion and exposed for 6 days. Slides were developed in Kodak D-19 developer and stained in 4% Giemsa (Harleco).

Fifty randomly chosen cells from each duplicate culture (total cell numbers = 100) were examined for the number of silver grains. The results from one post-UV incubation are shown in Table 1. Results from other post-UV incubation times (2 and 6 hr) were in agreement. BS cells exhibited about double the control levels of UV-induced UDS. These results suggest that BS cells: 1) may be more competent in recognizing thymidine dimers; 2) have increased exonuclease activity resulting in a larger repair patch size; or 3) have a smaller nucleotide precursor pool size than control cells.

TABLE 1

UV-INDUCED UDS IN BS FIBROBLAST CELLS

	Control	BS_1	BS_2
No. of grains* ($X \pm S_x$)	7.7 ± 0.4	15.6 ± 0.6	14.9 ± 0.6

*Corrected for background and control levels. ^{3}H-thymidine incubation was 4 hr. Control cell line, GM 498; BS_1, GM 1492; BS_2, GM 2520: all obtained from Human Genetic Mutant Cell Repository, Camden, NJ.

Chromosomal Aberrations Induced by Ionizing Radiation In G_2 PHA-Stimulated Peripheral Lymphocytes. Peripheral lymphocytes, cultured as described previously (10), were obtained from two BS-affected individuals and two normal controls. Cultures were exposed to gamma rays (Cobalt-60; dose rates of about 430 and 100 rads/min) two and one-half hours prior to culture termination. Colcemid (Gibco) was added immediately after irradiation. After routine termination and slide preparation, 50 metaphase cells were scored for chromosomal aberrations from each individual (10,11). After an exposure of 50 rads, BS metaphase cells exhibited approximately one-half of the number of chromatid-type aberrations observed in control cells (30 vs 60 achromatic lesions, 17 vs 37 chromatid deletions). In the second BS patient and after 100 rads, the difference, while not as great, was still highly significant (184 vs 326 achromatic lesions, 81 vs 135 chromatid deletions). These results suggest that BS cells may be more capable of repairing gamma-ray-induced DNA damage than are control cells.

DISCUSSION

The results presented here suggest that BS cells are not deficient in DNA repair, though this conclusion is still open to question. First, an examination of thymidine dimer removal and repair patch size in these same fibroblast cell lines (in conjunction with Dr. R. B. Setlow) has not detected any significant difference between BS and control cells, suggesting the difference may be due to nucleotide pool size. The observed decrease in chromosomal aberrations after exposure to gamma-rays could result from a significantly altered length of G_2. Experiments are in progress to differentiate these alternative possibilities. Though no decrease in DNA repair capability has been observed for BS cells (6,7), BS cells still appear to exhibit decreased cell survival when exposed to UV light. Also in conflict are the observations that while no deficiency in DNA polymerase activity has been observed for BS cells (8), DNA chain growth rate is depressed (5) and parental DNA piece size appears to be reduced (6). These observations are consistent with the hypothesis (9) that BS cells may constitute a pre-stressed population, as a consequence of endogenous production of an agent(s) capable of damaging DNA.

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SINGLE-STRAND DNA BREAKAGE AND REPAIR IN BLOOM'S SYNDROME CELLS[1]

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ABSTRACT The formation and repair of x-ray induced single-strand breaks in DNA was found to be the same in Bloom's syndrome and normal cells.

INTRODUCTION

Bloom's syndrome (BS) is a rare, autosomal recessive disorder characterized by reduced intrauterine and extrauterine growth leading to short adult stature, sensitivity to sunlight in childhood, facial telangiectasias, impaired immune function, and disposition to malignancy (1,2).

BS cells exhibit more than a tenfold greater frequency of sister chromatid exchanges (SCE's) than normal cells (3). They also exhibit quadriradial configurations, the result of chromatid exchanges involving homologous regions of homologous chromosomes. Quadriradial chromosomes are not commonly observed in normal cells, but may be induced in normal cells by treatment with the difunctional alkylating agent mitomycin C (MMC, 4).

The incidence of SCE's in normal cells may be increased by UV (5), x-ray (6), BUdR (7), and MMC (8) treatment, and the manifestation of SCE is S phase dependent (9). The first three agents stimulate recombination (10,11,12). Because SCE's can be induced by treatment early in G_1, the initial lesion responsible for SCE formation is relatively long-lived, and probably not a single-strand break (13). However, single-strand breaks probably are involved in meiotic crossing over (14), recombination (15), and chromatid exchange (16). These processes may each share some steps of the excision repair

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process which is operative in UV, x-ray, and MMC induced damage, and which results in the formation of single-strand breaks. In light of this, we have examined the extent of single-strand breakage induced by x-rays in BS cells, and also the capacity of BS cells to repair single-strand breaks in DNA.

RESULTS

The extent and kinetics of repair of single-strand breakage in DNA was assayed according to the protocol given in Fig. 1. The DNA from cells irradiated with 30 kR of x-rays with no opportunity for repair (Fig. 1, c) had a lower mean subunit size than DNA given 1 kR of x-rays (b). DNA from

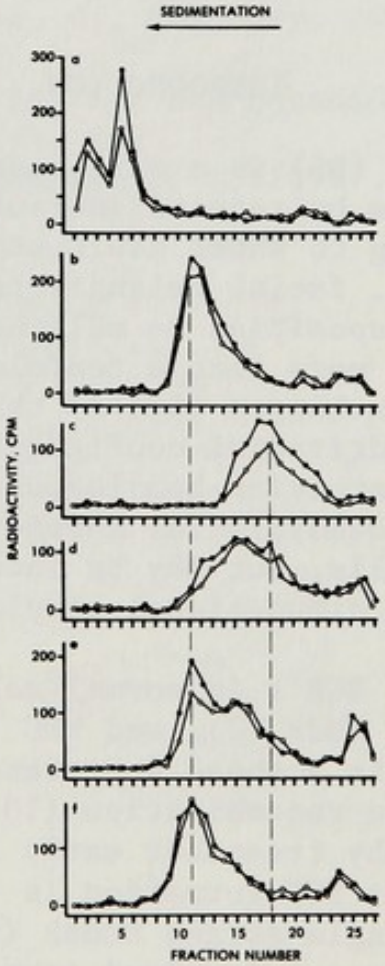

FIGURE 1. Alkaline sucrose gradient sedimentation of DNA from normal (SaJo, o-o) and BS (GM-1493, •-•) cells. DNA was labeled by incubating cells for 48 hrs in medium containing 0.1 μCi/ml [^{3}H]Thd (2 Ci/mM, SaJo) or 0.05 μCi/ml [^{14}C]Thd (0.053 Ci/mM, GM-1493). Cells were then x-irradiated (250 kVp, 15 mA, 1 mm Al filtration, 1.6 kR/min, 18.8 min, on ice), incubated for repair, irradiated with 1 kR of x-rays to reduce entanglement (all but a), scraped into suspension in Puck's balanced salt solution, and added to lysis buffer. Lysis was for 3.5 hrs at 24°C, and centrifugation was for 45 min at 38,000 r.p.m. in a SW 50.1 rotor. (a) no x-rays, (b) 1 kR, (c-f) 30 kR, with 0, 0.5, 1, and 2 hrs repair incubation.

TABLE I

DISTANCE CO-SEDIMENTED (MM) AS A FUNCTION OF REPAIR INCUBATION PERIOD FOR DNA FROM X-IRRADIATED NORMAL AND BS CELLS[a]

Culture	30kR				1kR	0kR
	Repair Incubation, hr					
	0	0.5	1	2		
GM-179	9.2	17.2	20.9	20.9	23.1	32.5
GM-1492 (BS)[b]	9.8	17.6	21.3	21.3	23.4	33.0
SaJo[b]	13.5	16.4	21.3	25.0	23.8	34.9
GM-1493 (BS)	13.7	18.1	21.6	24.0	23.4	35.2
GM-179[b]	11.3	18.9	23.0	24.6	25.1	37.2
GM-811 (BS)	12.5	18.1	21.8	25.0	25.0	36.7
SaJo	12.5	19.6	25.1	25.3	25.0	39.1
GM-1620 (BS)[b]	12.4	19.7	25.5	25.1	24.6	39.9
GM-316[b]	11.2	14.2	16.9	21.9	27.1	28.8
GM-2520 (BS)	11.2	17.1	21.8	23.1	29.3	27.5
SaJo	10.3	23.3	22.6	23.6	26.8	36.5
GM-179[b]	11.0	23.4	23.1	24.3	26.8	36.5
GM-179[b]	12.3	13.7	21.1	22.8	22.6	24.3
GM-316	12.0	13.4	20.6	23.1	23.3	24.8

[a]GM designations are for cultures of fibroblast-like cells obtained from the Human Genetic Mutant Cell Repository, Camden NJ 08103. SaJo was derived from a 20 year-old, normal female. The experimental protocol is given in the legend to Fig. 1.

[b]DNA labeled with [^{3}H]thymidine; other culture with [^{14}C]thymidine.

cells given 0.5, 1, and 2 hrs incubation for repair exhibited progressively larger mean subunit sizes (d-f).

The results of experiments with five BS and three normal cultures are summarized in Table I. In all cases, the distances sedimented for DNA from irradiated BS cells, with or without repair, were not different from those of co-sedimented DNA from normal cells.

DISCUSSION

The failure to detect differences in the sedimentation of DNA from irradiated BS and normal cells given no opportunity for repair is evidence that the number of single-strand breaks induced by 30 KR of x-rays was the same for BS and normal cells. This dose would be expected to form approximately 13 single-strand breaks and 1 double-strand break/2 x 10^{8} daltons DNA.

The similar kinetics of repair observed for the BS and normal cells suggests that BS cells, like normal cells, are able to repair large numbers of single-strand breaks in DNA, and also that BS cells are not likely to possess significantly greater numbers of unrepaired single-strand breaks than normal cells after 2 hrs incubation for repair.

These results imply that factors other than the functional capacity of ligation enzymes may be responsible for the BS defect. However, sedimentation in alkaline sucrose does

not differentiate repair from misrepair, and the increased frequency of SCE's and chromatid exchanges between homologous chromosomes seen in BS cells may arise from a misrepair process. The apparently normal rates of single-strand breakage repair in BS cells suggest that if a misrepair process is operative in BS cells, it may not be induced by a reduced rate of repair of single-strand breaks. The fate of the unrepaired single-strand breaks and the potentially lethal double-strand breaks in the BS and normal cells is also unknown, and BS cells may be less able than normal cells to cope with these or other x-ray induced lesions while exhibiting normal kinetics of repair for the majority of the single-strand breaks that are formed.

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EFFECTS OF DNA DAMAGING AGENTS ON CULTURED FIBROBLASTS DERIVED FROM PATIENTS WITH COCKAYNE SYNDROME

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ABSTRACT Cultured fibroblasts from Cockayne patients, as compared to those from normal subjects, exhibit increased sensitivity to UV, 4NQO or N-AcO-AAF but not to X-rays, EMS or ICR-170. Fibroblasts from four heterozygotes have intermediate UV sensitivities. Cockayne cells also incorporate ^{3}H-thymidine normally after UV irradiation.

INTRODUCTION

Cockayne syndrome (CS) is an autosomal recessively inherited disease characterized by cachetic dwarfism, retinal abnormalities, deafness, developmental and neural retardation after birth, microcephaly, and skin hypersensitivity to sunlight. Skin fibroblasts in culture derived from two CS patients were more sensitive to ultraviolet light (UV) but not to X-rays as compared to normal human fibroblasts.[1,2] A normal rate of excision of UV-induced pyrimidine dimers was observed in these CS cells.[2] This paper summarizes our recent study[3] on *in vitro* cell survival, as measured by colony forming ability, of eight additional CS fibroblasts strains, as well as fibroblasts from four parents, after exposure to several selected physical and chemical agents known to interact with DNA. These results, together with measurement of DNA repair synthesis after UV irradiation, indicate that the *in vivo* and *in vitro* cellular hyperphotosensitivity of this syndrome is due to a defect in DNA repair, but the precise molecular nature of the defect remains obscure.

RESULTS AND DISCUSSION

Figure 1 shows cell survival, as measured by colony forming ability,[2,3] of ten CS and two normal fibroblast strains after UV irradiation. All CS strains are markedly more sensitive to UV than normal human fibroblasts. Variation in UV sensitivity exists among different CS cells strains (e.g., strain NN), implying possible genetic heterogeneity in this

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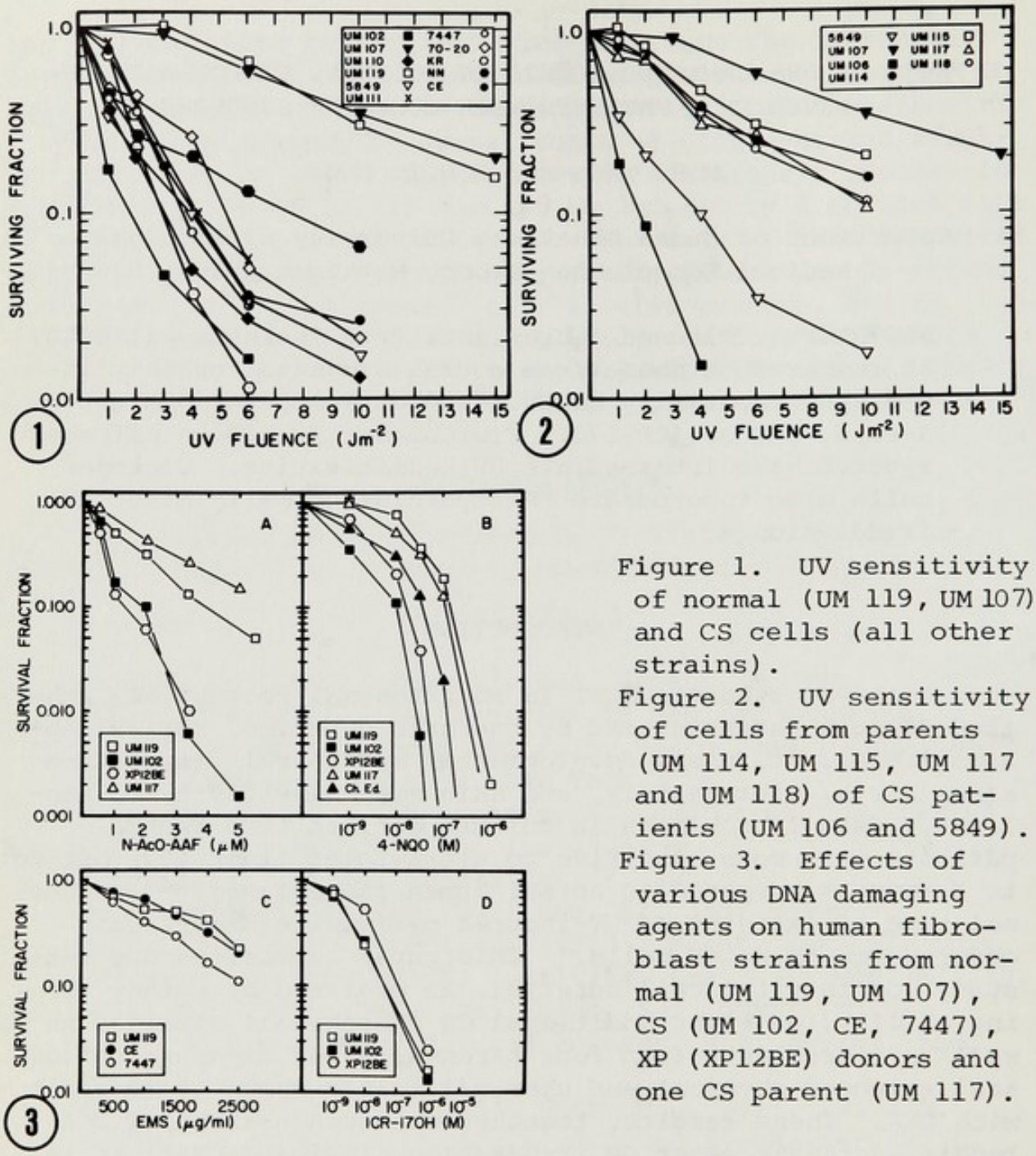

Figure 1. UV sensitivity of normal (UM 119, UM 107) and CS cells (all other strains).

Figure 2. UV sensitivity of cells from parents (UM 114, UM 115, UM 117 and UM 118) of CS patients (UM 106 and 5849).

Figure 3. Effects of various DNA damaging agents on human fibroblast strains from normal (UM 119, UM 107), CS (UM 102, CE, 7447), XP (XP12BE) donors and one CS parent (UM 117).

TABLE 1

^{3}H-THYMIDINE INCORPORATION INTO UV IRRADIATED CELLS DURING VARIOUS POSTIRRADIATION PERIODS

Cell Strain	Radioactivity [a] .5 hr	2 hr	3.5 hr
UM 119 (normal)	6.94	12.40	10.00
UM 102 (CS)	5.46	10.93	10.34
XP12BE (XPA)	0.97	0.86	0.86

[a] Radioactivity expressed as the ratio of CPM/μg DNA in irradiated over that in unirradiated cells.

syndrome. Cultured fibroblasts from parents of two CS children showed intermediate sensitivity to UV (Figure 2), although the parents were clinically asymptomatic. This finding differs from that of obligatory heterozygotes of Xeroderma pigmentosum (XP), whose cells in vitro have the same UV sensitivity as normal controls.[4]

Caffeine has been shown[5] to inhibit the post replicational repair process, presumably by binding to the single stranded DNA regions containing unexcised pyrimidine dimers. We found that the presence of .5 mM caffeine had no effect on the survival of two CS strains after UV irradiation, suggesting that the caffeine-sensitive, post-replicational repair process in these cells is probably normal.

Agents that are known to damage DNA with different modes of action were chosen to study their cytotoxic effects on CS cells. On the basis of the amount of DNA that is excised by the excision repair process, 4-nitroquinoline-1-oxide (4NQO), N-acetoxy-acetylaminofluorene (N-AcO-AAF) and UV have been considered[6] long patch repair inducers, whereas the acridine mustard ICR-170, X-rays, ethyl methanesulfonate (EMS) and perhaps 4NQO, short patch repair inducers. This division of agents into two classes may not be entirely valid [7,8], but it is not unreasonable to assume that similar types of DNA damage may be repaired by similar mechanisms.[9]

Figure 3A shows that when exposed to N-AcO-AAF the colony forming ability of CS and XP cells was more reduced than that of normal cells. Similar results have been obtained with 4NQO (Figure 3B). These observations support the view that CS cells are unable to undergo long patch repair process for damage caused by these agents.

Figures 3C and 3D show that CS cells survived with the same degree of sensitivity as normal cells to the cytotoxic action of ICR-170H and EMS. Our result would suggest ICR-170H as a short patch repair inducer, along with X-rays and EMS which also have similar killing effects on CS, XP and normal cells.

CS cells as well as normal cells, incorporated ^{3}H-thymidine into DNA after UV-irradiation, while this ability was significantly reduced in one XP cell strain tested, as expected (Table 1 and Ref. 3). Autoradiographyic studies with two CS cell strains yielded similar results, further supporting the apparent ability of CS strains to remove UV-induced pyrimidine dimers with endo- and exonucleases and to fill in the gap with a DNA polymerase. Assays of partially purified cell extracts for DNA polymerase show similar values for normal and Cockayne strains (Wade, unpublished).

In summary, these results show that CS cells are clearly more sensitive than normal to long patch repair inducer types

of DNA-damaging agents, but caffeine-sensitive post replicational repair, removal of UV-induced pyrimidine dimers and polymerization of polynucleotides in the damaged region are apparently intact in these cells. Furthermore, the resealing process necessary for repair of damage caused by EMS, ICR-170 and X-rays also appears normal.

Several possible explanations may be considered. First, a mutant polymerase which inaccurately inserts bases during unscheduled DNA synthesis could show a normal level of ^{3}H-thymidine incorporation but lead to cell lethality. If this is the case, infidelity of DNA repair would result in a high rate of mutation in CS cells. A separate study in our laboratory shows that the frequency of spontaneous and UV-induced mutations in CS cells are comparable to normal cells[10]. The second possibility is that CS cells possess a defective ligase specific for long patch repair, which suggests that more than one ligase must exist in human cells since CS cells appear to be fully capable of resealing newly synthesized DNA strands during repair of damage inflicted by EMS, ICR-170H or X-rays. Finally, the possibility must be considered that some unknown co-factor or enzyme needed for repair of UV, 4NQO or N-Aco-AAF induced damage is defective or absent in CS cells.

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SISTER CHROMATID EXCHANGES IN DYSKERATOSIS CONGENITA AFTER EXPOSURE TO TRIMETHYL PSORALEN AND UV LIGHT[1]

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ABSTRACT Exposure in vitro of cultivated peripheral leukocytes to trimethylpsoralen and UV light (365nm) under conditions that are known to produce DNA cross-links caused a greater increase in the number of sister chromatid exchanges in cells from two patients with dyskeratosis congenita (DKC) than in normal cells. Absence of this enhancing phenomenon in an unaffected sibling of one patient as well as its presence to a partial degree in an obligate carrier suggest that the observation may be etiologically significant. The data suggest that DKC is associated with a heritable defect in the repair of DNA cross-links.

INTRODUCTION

Dyskeratosis congenita, first described by Zinser in 1910 (1), is a rare genodermatosis. Its chief integumental characteristics are mucosal leukokeratosis, nail dystrophy and a patterned telangiectatic hyperpigmentation. Associated non-cutaneous problems such as malignancy and infections are severe and often lead to early death. Most patients have pancytopenia. In Fanconi's anemia (2), which is sometimes confused with dyskeratosis congenita, chromosomes have frequent breaks and rearrangements. Chromosomes of patients with dyskeratosis congenita are usually normal, but chromosomal instability has been suggested by isolated reports of endoreduplication, gaps and increased sister chromatid exchanges (3). Most patients with dyskeratosis congenita are males and the disease appears to be inherited as an X-linked recessive disorder. The cause has not yet been found.

Sister chromatid exchanges (SCEs) are sensitive indicators of chromosomal injury caused by mutagens and carcinogens (4).

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TABLE I: SISTER CHROMATID EXCHANGES IN HUMAN LYMPHOCYTES (SCEs/CELL)

		Pt. With Dyskeratosis Congenita	Normal Brother Of Patient	Normal Control	Mother Of Pt.
A.	Untreated	9.44 (±1.71)	10.20 (±2.12)	9.67 (±1.45)	--
	TMP alone[a]	11.70 (±1.16)	--	--	--
	UV-A alone[b] (0.9 J/cm^2)	9.40 (±2.38)	--	--	--
	TMP plus UV-A (0.3 J/cm^2)	22.72 (±2.79)	--	--	--
	TMP plus UV-A (0.6 J/cm^2)	32.88 (±3.90)	--	--	--
	TMP plus UV-A (0.9 J/cm^2)	44.53 (±6.74)	30.20 (±4.53)	30.50 (±4.07)	--
B.	Untreated	9.5 (±1.4)	--	9.8 (±1.6)	10.1 (±2.1)
	TMP plus UV-A (0.6 J/cm^2)	37.8 (±6.8)	--	23.5 (±2.5)	30.7 (±6.0)

[a] TMP is 4, 5' 8 trimethylpsoralen. Irradiations performed in vitro.

[b] UV-A is UV light (365nm).

Though not fully understood, SCEs may represent a DNA repair process (5). Chemical agents that produce DNA cross-links promote SCEs more readily than do those that cause other kinds of DNA damage.

The psoralens are photosensitizing furocoumarins used in the treatment of vitiligo and psoriasis. Covalent binding of psoralens to DNA is photomediated by long-wave UV light (365nm). Monofunctional and bifunctional photoadducts are formed depending upon whether a single psoralen molecule binds to one strand or to both strands of DNA. Trimethylpsoralen (TMP) in the presence of UV light produces TMP-DNA cross-links (6,7).

MATERIALS AND METHODS

Peripheral blood leukocytes from two unrelated persons with dyskeratosis congenita (DKC), their mothers, an unaffected brother of one person with DKC and normal controls were cultivated in McCoy's medium with phytohemagglutinin. After 24 hours, some cells were exposed to 4, 5' 8 trimethylpsoralen (TMP) and/or UV light (UV-A, 365nm). All flasks received BrdU (20ug/ml) for 48 hours and colchicine, 3 X 10^{-6}M for 90 min. Spreads of mitotic chromosomes were prepared, stained with Hoechst 33258 and Giemsa, and the number of sister chromatid exchanges (SCEs) was determined per metaphase (6).

RESULTS

Exposure to TMP and UV-A increased the number of SCEs in lymphocytes from normal persons and in two patients with dyskeratosis congenita (DKC). The increase in SCEs was dependent upon the amount of both psoralen and UV light in cells from DKC. At equal concentrations of TMP and identical exposure to UV light the increase in SCEs was consistently greater for DKC cells than for similarly treated normal cells. Treatment of cells with psoralen alone or UVA alone did not affect the SCE rates in cells from DKC or normal cells. The 12 year old brother of one patient with dyskeratosis congenita was clinically normal and treatment of his cells with TMP and UV light produced the same enhancement of SCEs as with cells from unrelated, normal controls. An intermediate, elevated value in SCEs was found after treatment of cells from the mother of the patient.

DISCUSSION

Dyskeratosis congenita is associated with malignancy. Certain other diseases, which also have a propensity toward

malignancy have been associated with defects in DNA repair (1, 4, 7). We have reported elsewhere that fibroblasts cultivated from patients with dyskeratosis congenita (DKC) are slow to remove photomediated, psoralen-DNA cross-links (8). SCEs may reflect poorly-repaired DNA damage and the heightened production by psoralens of SCEs in DKC cells suggests either that more psoralen-DNA photoadducts were formed or that fewer photoadducts were removed by the DKC cells compared to normal cells. The present data do not distinguish between these two possibilities. The normal SCE rates of the clinically normal brother imply that his cells deal with psoralen-DNA photoadducts differently than do the cells from his affected sibling. The intermediate value in SCEs from treated cells in the clinically normal mother probably reflects her carrier state. Our data are consistent with the concept that a defect in the ability to repair DNA cross-links is a fundamental abnormality in dyskeratosis congenita.

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FANCONI'S ANEMIA
A CONDITION POSSIBLY ASSOCIATED WITH A DEFECTIVE DNA REPAIR[1]

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ABSTRACT Fanconi's anemia (FA) raises a special genetic interest, since it has been associated with the increased chromosomal instability and strong predisposition for the development of cancers. Particularly, the last few years have given us new fundamental insight into the biochemical basis of FA. Of significant finding is that the FA cells are highly sensitive to cytotoxic and chromosome breaking action of bifunctional alkylating agents. The cells appear to have reduced ability to repair DNA interstrand cross-links. Other works show limited capacity to repair γ- and UV-induced damage produced at relatively high dose. These observations might be due to the inherent defect in a function that is required for the DNA interstrand cross-links or closely spaced overlapping damage to be repaired.

INTRODUCTION

FA, or Fanconi's syndrome of pancytopenia and multiple anomalies, first described by Fanconi (1) in 1927, is a rare genetic disease transmitted as an autosomal recessive trait (2). The FA homozygote is identified by the combination of growth retardation, pigmentation anomaly of the skin and diverse congenital anomalies associated with a progressive insufficiency of the bone marrow leading to pancytopenia of the peripheral blood. Frequent association with leukemia and other solid tumors is sufficient to indicate that this condition strongly predisposes to the development of cancers (3-5). The cancer risk is also increased in relatives of the affected persons, presumably heterozygous for FA gene (6). The skin fibroblasts from FA patients have been demonstrated to be more readily transformed by oncogenic virus SV40 in tissue culture than those from normal persons (7-9). Since Schroeder *et al.* (10) discovered the increased frequency of chromosome structural aber-

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rations in peripheral blood lymphocytes, FA has won a special genetic interest and stimulated the recent works on the biochemical defect associated with the FA gene. Our current knowledge is now in line with the idea that the FA may be categorized into a special class of genetic disorder that has an impairment in a function relating somehow to the repair of certain types of DNA damage, probably DNA interstrand crosslinks as well as closely spaced overlapping damage.

DIVERSITY IN PHENOTYPIC EXPRESSION OR GENETIC HETEROGENIETY

Diversity in phenotypic expression and genetic heterogeniety are our general experience in medical genetics. Although there are many cases in which clinical symptoms are sufficient to identify FA, this condition also presents a wide spectrum of clinical changes, which often leads us to the difficulties in establishing the diagnosis. A tendency towards the intrafamilial clustering of similarly affected patients and correlation between age at onset and the severity of malformations suggest genetic heterogeniety (4,10). However, clinical characteristics are not always sufficient to indicate so. It has been proposed (11) that the chromosomal instability, which is present even before the onset of anemia (12) and is probably closer to the basic defect, is a leading symptom for establishing the diagnosis of FA. Table I summarizes clinical and cytogenetic data on patients

TABLE I
SUMMARY OF CLINICAL AND CYTOGENETIC DATA ON FA PATIENTS

FA-JTO	Sex	Age (yr)	Consanguinity	Microsomy	Pigmentation	Thumb anomaly	Hypogonadism	Hyperreflexia	Microcephaly	Ocular anomaly	Kidney anomaly	Other anomaly	Mental defect	Chromosomal instability: Sponta. (%)	Relative sensitivity: Ratio of induced aberration yield relative to that in normal cells: γ-ray	UV	4NQO	DCMMC	MMC	MMS	MNNG	
FA1	M	6	+	+	+	+	+	+	-	-	-		-	60.5	--	--	--	--	--	--	--	
FA2	F	6	-	+	+	-			-	-	-		-	67.5	--	--	--	--	--	--	--	
FA5	F	6	-	+	+	+		+	-	-	+	+	-	76.5	1.1	7.0	1	--	41.8	1	1	
FA6	F	3	+	+	+	+		-	-	-			-	91.0	1.1	--	--	--	112.9	--	--	
FA7	M	4	+	+	+	+	+		-	-	-	+	-	54.0	--	--	--	6.2	27.2	--	--	
FA9	M	7	+	±	+	+	+	+	-	-	-		-	87.0	1	--	--	--	67.1	1	--	
FA10	F	8	-	+	-	-		-	-	-	-		+	13.0	--	1	--	1	3.9	--	--	
FA12	M	3	+	+	+	+	+	+	+	+	-	+	-	57.0	--	--	--	--	33.2	--	--	A
FA13	F	3	+	+	+	-			-	-	-			39.5	--	3.6	--	3.6	27.0	1.2	--	
FA14	M	8	-	+	+	-	+	-	-	-	-		-	50.0	--	--	1	--	18.6	1	--	
FA17	M	11	-	±	+	-	+	-	-	-	-		-	39.0	--	--	--	--	9.0	--	1	
FA18	M	7	-	+	+	-	+		-	-	-		-	63.0	--	1	--	1.0	28.4	1	--	
FA20	M	11	+	+	+	+	+		+	-	-	+	-	40.0	--	1.2	1.3	--	11.9	1.3	--	
FA21	M	6	-	±	+	-			-	+	+		-	42.0	--	--	1	--	7.2	1	--	
Average														55.7	1.1	2.8	1.1	2.9	32.3	1.1	1	
FA3	F	3	-	+	-	-			-	-	-		+	6.0	--	--	--	--	--	--	--	
FA4	F	4	-	+	-	-				-	-		+	3.0	--	--	--	--	--	--	--	
FA8	F	4	-	+	-	-		-	-	-	-	+	+	6.5	--	1.3	--	--	1.5	1.6	--	
FA11	M	2	-	+	-	-	-		-	-	-			4.0	--	1.4	--	--	1.1	--	--	B
FA15	F	5	-	+	±	-			-	-	-		-	9.0	--	--	--	--	1	--	--	
FA19	F	7	-	-	-	-		-	-	-	-	+	+	7.0	--	--	--	--	1	--	--	
Average														5.9	--	1.4	--	--	1.1	1.6	--	

clinically suspected to be FA because of their constitutional aplastic anemia occuring in association with growth retardation and/or malformations. Cytogenetic data clearly divide these into two groups; group A having high frequency of chromosome aberrations and group B showing normal level of spontaneous chromosome aberrations. A question remains to be answered as to whether the patients in group B are either variant form or they are categorized into entirely different conditions. They lack some of the typical symptoms such as pigmentation anomaly, malformation of thumb and underdevelopment of gonad (in boys).

CYTOGENETIC CHARACTERISTICS

Spontaneous Chromosome Aberrations. Assuming that the basic unit of interphase chromosome is a single DNA duplex, any interruption in the continuity of chromosome may directly or indirectly be related with the basic DNA metabolism such as replication, recombination and repair. The cytogenetic phenotype may thus provides us an important key to the understanding of the basic defect in FA. The chromosomal instability in FA is characterized by an elevated level of spontaneous chromosome aberrations. However, the spectrum of aberrations differs in many respects from those found in other chromosome breakage syndrome, *e.g.*, Bloom's syndrome and louis-Bar's syndrome. The chromosome aberrations in FA are mainly open breaks or gaps of the chromatid type (Fig. 1a). Chromatid exchanges are rather infrequent, and when occurred, in a rather random combination of non-homoloques. Occasional endoreduplication mitosis (Fig. 1b) and extensive fragmentation (Fig. 1c) are also characteristic

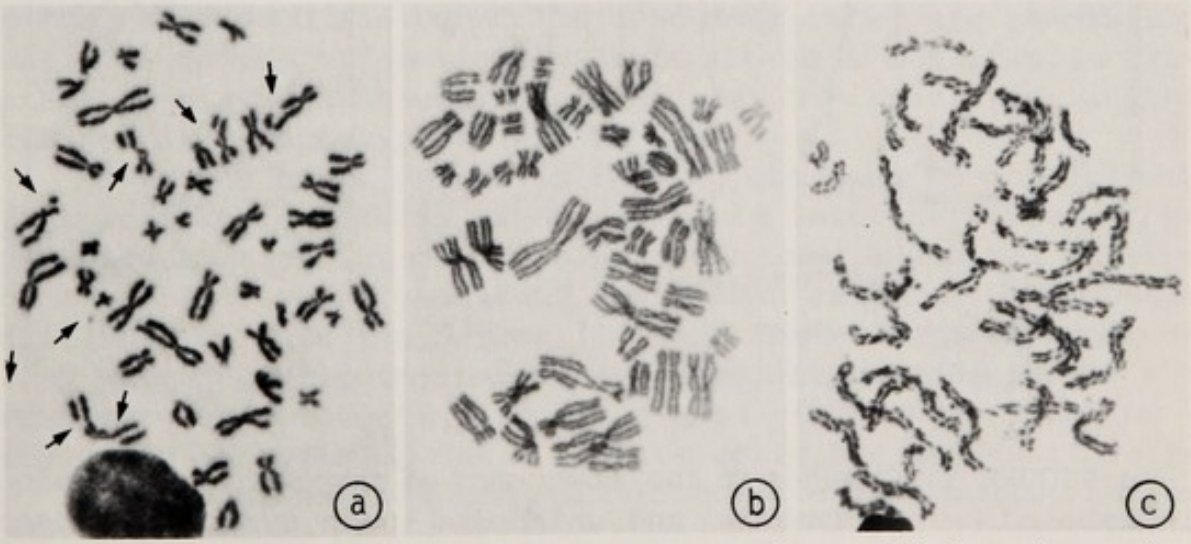

FIGURE 1. Spontaneous chromosome abnormalities in peripheral blood lymphocytes from FA patients. a, Diploid cell with chromatid breaks and gaps (arrows); b, Tetraploid cell showing endoreduplication; c, Cell with extensive fragmentation.

to FA. Unlike Bloom's syndrome, in which manyfold increase in the sister chromatid exchange (SCE) has been noted (13), FA shows a normal level of spontaneous SCE rate (13,14).

Chromosomal Sensitivity to Mutagens. In relation to the basic defect in FA, of considerable interest may be a mutagen specific sensitivity to chromosome breakage. High sensitivity of FA to chromosome breakage, first noted by Schuler *et al.* (15) in cultured lymphocytes treated with tetrasulfonyl-d-mannit, now seems to be specific to the agents that can introduce interstrand crosslinks into DNA such as mitomycin C (MMC), nitrogen mustard (HN2) and psoralen-plus-light reaction (16). Exposure to agents that do not induce crosslinks, *e.g.*, γ-ray, UV, methyl methanesulfonate (MMS), 4-nitroquinoline 1-oxide (4NQO) and decarbamoyl mitomycin C (DCMMC), reveals little difference in induced aberration frequency between normal and FA cells. In Table I are also listed the relative chromosomal sensitivities of FA patients as tested in their peripheral blood lymphocytes. The high sensitivity to MMC seems to be a general feature of FA, suggesting some impaired capacity to tolerate interstrand crosslinks. However, it should be noted that the cells from patients with normal level of spontaneous chromosome aberrations (group B) are also normal in the response to MMC.

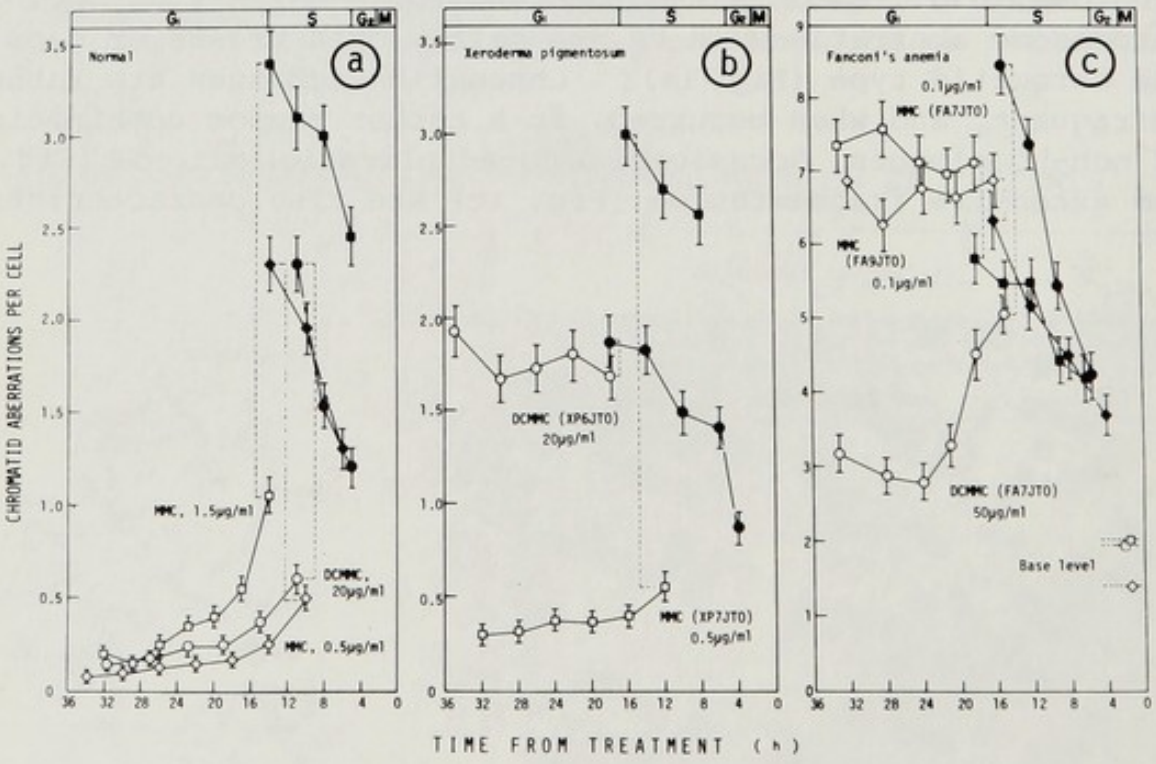

FIGURE 2. Changes in the frequency of chromatid aberrations in labeled (solid symbols) and unlabeled (open symbols) mitoses. At the indicated time before harvest, lymphocyte cultures were treated with chemicals together with ^{3}H-thymidine for 30 mins and reincubated in normal fresh medium. a, Normal cells; b, XP cells; c, FA cells.

Another chromosomal response that might be relevant to the basic defect of FA is a markedly reduced increment of the SCE formation by MMC while such is less obvious in case of treatment with ethyl methanesulfonate (EMS) (17,18).

Repair of Preaberration Lesions. The impaired capacity of FA to repair DNA crosslinks is more highly suggestive in the cytogenetic assay for the loss of preaberration lesions (19,20). DNA damage may link to the formation of chromosome aberrations when they pass through the DNA synthesis (S) stage. Therefore, the aberration yield may be related with the amount of damage that has not been repaired before the damage-bearing DNA enter S phase. In the repair deficient cells, one would expect a constant aberration yield whether the damage is induced away from or close in time to the S phase whereas repair proficient cells show a decrease in aberration yield when the damage is induced remote from the S phase. In such cytogenetic assay, FA cells show a response of the repair deficient type to MMC while they follow repair proficient type response to monofunctional DCMMC (Fig. 2). It is interesting in regard to the repair of MMC damage in human cells that xeroderma pigmentosum (XP) cells show the response of the repair proficient type to bifunctional MMC and repair deficient type to monofunctional DCMMC.

SENSITIVITY TO THE CYTOTOXIC ACTION OF MUTAGENS

When assayed by the inactivation of colony forming ability of cultured skin fibroblasts, FA cells show markedly high sensitivity to MMC (20-23). Figure 3 shows the survivals of FA cells after exposure to various mutagens. FA cells are as sensitive as normal cells to γ-ray, but some FA strains show slightly elevated sensitivity to UV and other monofunctional agents such as MMS, EMS, 4NQO and DCMMC (20). However, they are remarkably sensitive to the bifunctional agents like MMC, busulfan (tetramethylene bis-methanesulfonate) and near UV irradiation in the presence of 8-methoxypsoralen (8MOP).

As to the basic defect in FA, the changing susceptibility of human cells to MMC is of considerable interest since rather increased sensitivity to MMC also seems to be a general feature for another class of genetic disease such as familial polyposis coli (PL), Gardner's syndrome (GS) and Peutz-Jeghers' syndrome (PJ), which constitute dominantly transmitted condition of hereditary adenomatosis associated with high cancer risk (my previously unpublished data). Those cells are as sensitive to MMC as those of established cell lines like HeLa cells, but an order of magnitude less sensitive than FA cells and significantly more sensitive than normal cells (Fig. 3e). The inherent defect may be entirely different between these two classes of disease, FA and hereditary adenomatosis, and these results point to the complexity of regulatory mechanisms to tolerate MMC damage.

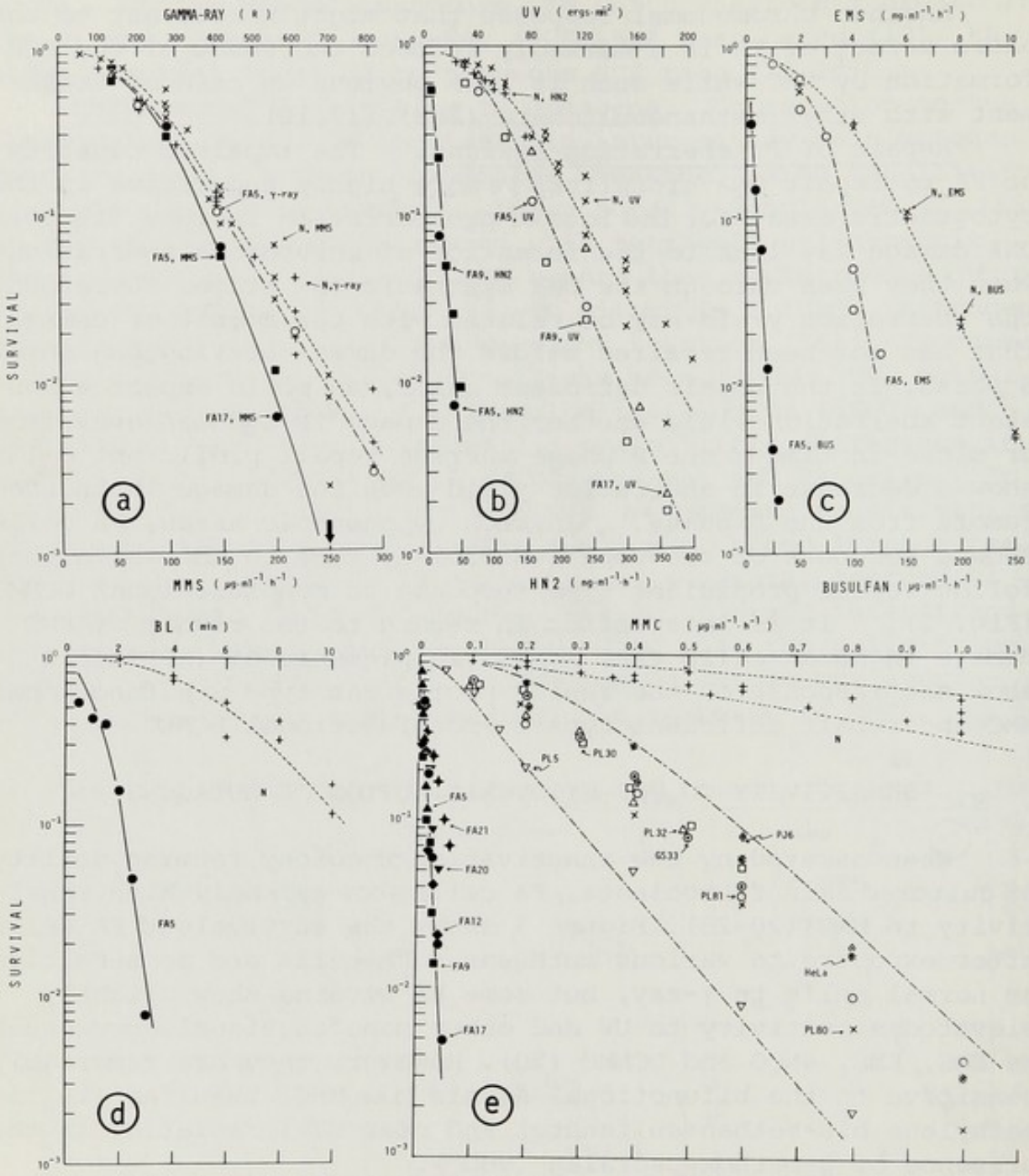

FIGURE 3. Comparison of survivals of normal (N) and FA fibroblasts after exposure to: a, γ-ray, MMS; b, 245nm UV, HN2; c, EMS, Busulfan; d, exposure to 355nm black light (BL) in the presence of 8MOP (1μg/ml); e, MMC (For PL, GS and PJ, see text).

FA IN DEFECTIVE DNA REPAIR

The abnormal sensitivity of FA cells to the cytotoxicity and chromosome aberration formation specifically by bifunctional alkylating agents strongly points to the impaired capacity to repair DNA interstrand crosslinks. This has been attested by Fujiwara *et al.* (22,23). In their assay of DNA from MMC-treated cells for its ability to release the covelent linking of duplex,

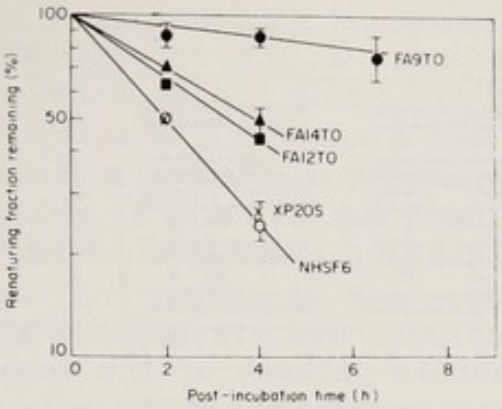

FIGURE 4. Rate of crosslink removal as calculated from hydroxyapatite chromatography for reversibly biherical DNA from MMC-treated normal human (NHSF6), XP and FA cells. Fujiwara (23).

the FA cells have been clearly demonstrated to be defective in the half-excision step, which constitutes the first step in the crosslink repair, while this step is readily identified to occur in normal and XP cells of complementation group A (Fig. 4). The defect may be in its first incision step or in its trigger function.

It has also been suggested that FA might have reduced exonuclease activity since UV-irradiated FA cells showed a reduced capacity in removing thymine dimer-containing part of DNA and hence in the final rejoining of resynthesized strand (24). However, other works show successful excision of thymine dimers (25) towards the completion of strand rejoining (23) in UV-irradiated FA cells. Remsen & Cerutti (26) reported that the sonicated preparations from some FA cells showed reduced ability to excise γ-ray products of the 5,6-dihydroxydihydrothymine type (t') from exogeneous DNA. More recently, using host-cell reactivation technique, Rainbow & Howes (27) found that FA cells had reduced capacity to support the infecting UV- and γ-ray-irradiated virus. Of particular interest is that all in these experiments the reduced repair capacity becomes apparent when DNA damage is increased to relatively high level. Slightly increased cytotoxicity to monofunctional agents might be relevant to this. FA cells are capable of rejoining X- or γ-ray-induced DNA strand breaks (24,28), suggesting the presence of ligase function. Normal activity of apurinic endonuclease has also been found in the extract from FA cells (29)

At present, it is still premature to pinpoint the location of the basic defect in FA. However, assuming that a single molecular deficiency is responsible for these line of observations, it is tempting to assume as a testable proposition that the FA cells might have some deficiency in the repair of closely spaced overlapping damage produced on the complementary DNA strands (Fig. 5). Such a possibility has also recently been suggested by Cleaver (30). Interstrand crosslink is a typical

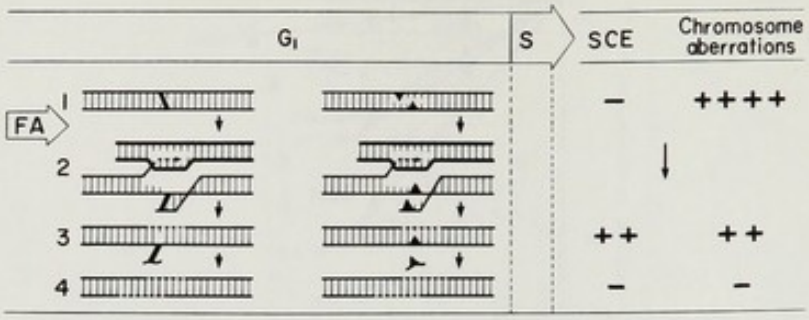

FIGURE 5. Possible pathway of the repair of interstrand crosslink and overlapping damage and its relevance to the chromosomal consequences. Possible site of defect in FA is indicated.

example of such damage, and other overlapping damage may depend on the total amount of damage as well as on the DNA base sequences.

Little has been known about the repair of crosslinks in human cells. However, to our knowledge available at present, the first half-excision step seems to be independent from the excision ability for pyrimidine dimers, which is diminished in XP (22,23). This is in marked contrast to the crosslink repair suggested in *E. coli*, in which it occurs in a sequential two-step process involving *uvrA* (or *B*) dependent excision of one side of crosslink followed by *recA* dependent exchange between homologous duplexes (31-33). Since MMC does not invoke detectable amount of unscheduled DNA synthesis in XP cells (20) in spite of their successful half-excision of crosslink (22,23), the unhooking may be a short-patch repair process. It has been demonstrated in mouse L cells that the unhooking is very rapid process with a half-life of 2h whereas the crosslink moiety still attached to the other strand is long lasting (34). As to the crosslink repair, there is an indication that the *E. coli* enzyme by itself can not repair crosslinks in DNA of infecting phage although it can repair those damage in their own DNA (35). Similarly, Day *et al.* (36) suggested that even normal human cells could not repair psoralen-mediated crosslinks in the DNA of infecting adenovisus. These lead us to a proposition, although highly speculative, that the repair of crosslinks or overlapping damage can only proceed in chorus with recombination between duplexes, which constitutes a triggering function, and that FA might be a condition relating to the deficiency in this function (Fig. 5). Severe meiotic failure found in FA is particularly interesting in this respect.

Giving a central role to the replicational and damage-tolerating mechanism in the SCE formation (37), crosslinks may not be tolerated by SCE mechanisms when they attempt to pass through DNA replication and hence giving rise to the reduced increment of MMC-induced SCE in FA cells (17,18), but efficiently link to the formation of chromosome aberrations. Half-excised

crosslink moiety still attached to the other strand might be a major source of MMC-induced SCE in normal cells (Fig. 5).

In *E. coli*, interstrand crosslink has been shown to be inmutable (38), and induced mutagenesis has been related with the *rec* and *lex* dependent inducible responses (39). FA cells show reduced production of ouabain and 6-thioguanine resistant mutants (40). This renews a perennial question as to why FA predisposes to cancer and points to the complexity in the relationship between mutagenesis and carcinogenesis.

ACKNOWLEDGMENT

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X-RAY SENSITIVITY OF FIBROBLASTS FROM PATIENTS WITH RETINOBLASTOMA AND WITH ABNORMALITIES OF CHROMOSOME 13[1]

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ABSTRACT Fibroblasts from patients with hereditary retinoblastoma appear to be more sensitive to killing by X-rays than do those from sporadic retinoblastoma or normal control patients. One of two D-deletion retinoblastoma strains was highly X-ray sensitive; normal sensitivity was restored when this strain was transformed with SV-40 virus. Preliminary evidence is presented to suggest X-ray sensitivity may be related in this case to a deletion in a specific region of chromosome 13.

INTRODUCTION

Retinoblastoma is a rare malignant eye tumor which may appear sporadically or occur in genetically predisposed persons. The tumors in the latter group, referred to as the hereditary type, are usually bilateral. Rarely, retinoblastoma may be associated with a specific deletion in the long arm of chromosome 13 which occurs in many somatic cells (D-deletion type). When this is present, the patients bear many of the defects of the D-deletion syndrome including microcephaly, microphthalmia and genitourinary abnormalities. Survivors of the hereditary type of retinoblastoma have a significantly increased risk for the development of other tumors both at distant sites and particularly within the irradiated field following radiation therapy.

We have recently reported the unusual X-ray sensitivity in vitro of skin fibroblasts derived from a patient with D-deletion retinoblastoma (1); at this time we noted that cell strains from 2 patients with hereditary retinoblastoma also appeared slightly more radiosensitive than normal (1). The present investigation was designed to extend this study to additional retinoblastoma strains (2), as well as to cells from patients with other abnormalities of chromosome 13.

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MATERIALS AND METHODS

Cell strains identified as GM or AG were obtained from the cell bank at the Institute for Medical Research, Camden, New Jersey. Other cell strains were obtained by us from various sources. All cells were grown in Eagle's MEM with 15% fetal calf serum as previously reported (1). Plating efficiencies generally ranged between 5-25%. Cells were irradiated with 220 kV X-rays while in exponential growth 18 hours after seeding at low concentrations; the techniques for survival experiments have been described in detail (1,2). All strains were studied between the 4th and 26th mean population doubling in culture. The $D_o \pm 1$ S.E. (inverse of the slope of the survival curve) was obtained for each cell strain by least square linear regression analysis of the pooled data from 3 or more separate dose-response experiments.

RESULTS

The survival data for skin fibroblast cell strains isolated from 4 patients with sporadic retinoblastoma and 5 patients with hereditary retinoblastoma are tabulated in Table 1. The D_os for the sporadic cases ranged from 135-164

TABLE I
X-RAY SENSITIVITY OF FIBROBLASTS
FROM RETINOBLASTOMA PATIENTS

Strain	Source	# Experiments	D_o (rads)
Sporadic			
RBCH	CHMC	3	164 ± 7
AG 1946	IMR	3	141 ± 7
HL 23B	MEEI	3	144 ± 7
GM 1979	IMR	3	135 ± 8
Hereditary			
GM 1123	IMR	3	121 ± 4
GM 1980	IMR	3	140 ± 8
GM 1879	IMR	5	128 ± 9
AG 1408	IMR	3	119 ± 5
GM 1880	IMR	6	98 ± 6

rads; this is similar to a number of normal strains studied in our laboratory. The D_os for the hereditary cases ranged

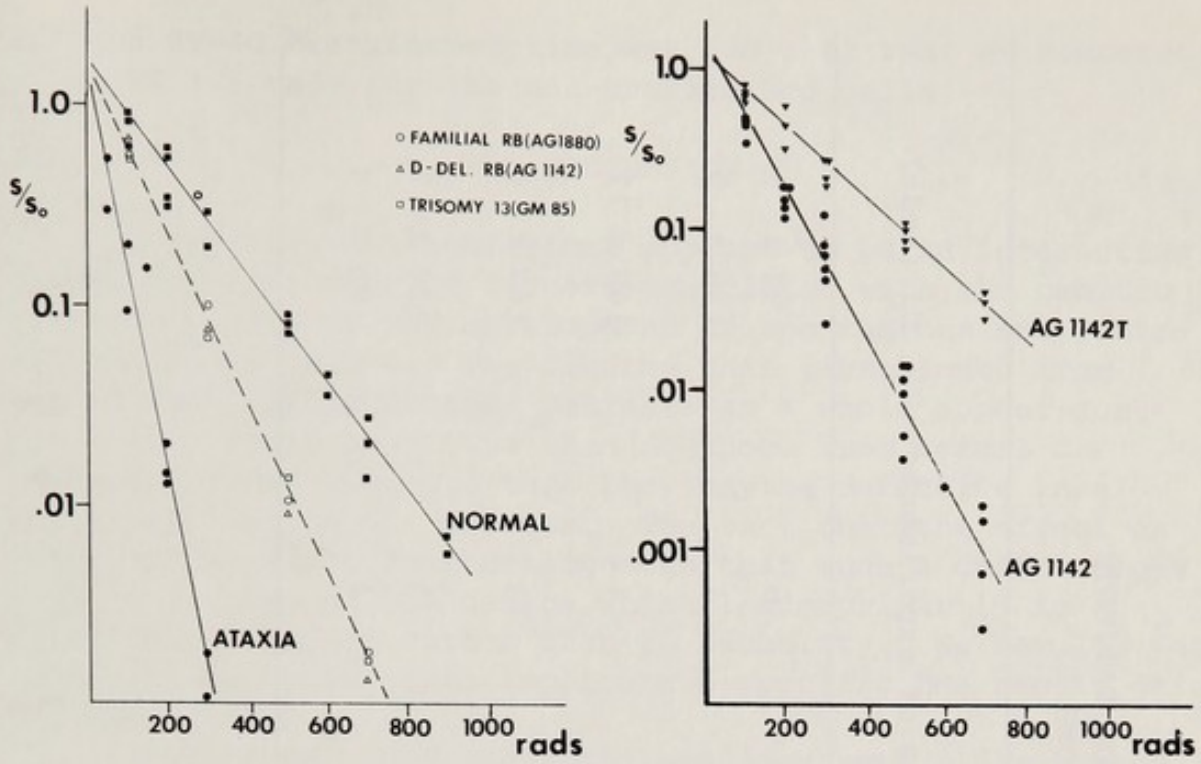

FIGURE 1. X-ray survival curves for several strains of human diploid fibroblasts (Tables 1 and 2).

FIGURE 2. Survival curves for D-deletion retinoblastoma strain (AG1142) before and after transformation by SV-40 virus.

from 98-140 rads. As a group these strains appear slightly more sensitive than the sporadic group; GM 1880 is clearly abnormally radiosensitive. This patient has a strong family history of multiple cancers including retinoblastoma.

The survival data for skin fibroblasts from patients with several different abnormalities of chromosome 13 are shown in Table 2. Cells from one D-deletion retinoblastoma and from a trisomy-13 were unusually radiosensitive, whereas cells from another D-deletion retinoblastoma (LKR) were normal in their response. The data for these 2 sensitive strains are plotted in Figure 1 along with data for an ataxia telangiectasia strain (CRL 1343, ATCC), a normal strain (Li 106), and the sensitive hereditary retinoblastoma strain (GM 1880, Table 1). Clearly these 3 sensitive strains are very similar in their response, and fall into a range intermediate between normals and the highly X-ray sensitive ataxia cells.

We previously reported (3) that the DNA repair capacity of a repair deficient progeric fibroblast strain was apparently restored after transformation by SV-40 virus. We therefore examined the X-ray sensitivity of the sensitive D-deletion retinoblastoma strain (GM 1142, Table 2) after SV-40 transformation. The results are shown in Figure 2. The D_o

TABLE 2
X-RAY SENSITIVITY OF FIBROBLASTS FROM PATIENTS
WITH ABNORMALITIES OF CHROMOSOME 13

Strain	Disease	Chromosome abnormality	# of experiments	D_o (rads)
GM 1142	Retinoblastoma	Interstitial deletion	6	94 ± 5
LKR	Retinoblastoma	Interstitial deletion	4	136 ± 9
LIZ	Coloboma	Terminal and interstitial deletion	4	149 ± 11
GM 509	D-deletion syndrome	Terminal deletion	3	147 ± 6
GM 729	--	Ring 13 (W.terminal deletion)	3	136 ± 5
GM 85	--	Trisomy 13	4	95 ± 4

of the SV-40 transformed line was 188 ± 11 rads as compared with 94 ± 5 rads for the non-transformed cells.

DISCUSSION

Hereditary retinoblastoma appears to be an interesting model for the study of factors associated with the genetic predisposition to the development of spontaneous and radiation-induced cancer. The finding that fibroblasts from hereditary retinoblastoma patients as a whole appear somewhat more X-ray sensitive than do those from normal or sporadic cases suggests that they may be defective in a molecular DNA repair process. The fact that the effect on survival is small further suggests that such a defect might involve a type of DNA damage which leads primarily to mutational changes rather than to lethality. We have as yet, however, no evidence to implicate a specific DNA repair defect in these cells.

D-deletion retinoblastomas are very rare (15 reported cases); these patients usually die early as a result of congenital abnormalities. It is thus not known whether they are genetically predisposed to the development of other tumors. Our results to date with D-deletion retinoblastoma and other cells with deletions of chromosome 13 (Table 2) show no apparently consistent pattern. From karyotypic analyses, however, the one region of chromosome 13 which is absent in the radiosensitive case (GM 1142), but present in all the others, is the central region of the light-staining band 13q14. A deletion involving the 13q14 band is common to all D-deletion retinoblastomas which have been examined. The possibility is interesting that a genetic locus may be present within the central region of this band which influences the expression of an X-ray-induced DNA repair process. Obviously, additional cases with deletions of portions of chromosome 13 must be studied in order to support this hypothesis.

We have no explanation at present for the apparent restoration of normal X-ray sensitivity in the SV-40 transformed D-deletion strain, but are currently extending this study to other X-ray sensitive and repair deficient cell strains. This finding is reminiscent, however, of our previous observation (3) that SV-40 transformation apparently restored the repair capacity of a repair deficient progeric strain, and suggests that viral transformation may influence the expression by the cell of normal DNA repair processes.

The results reported here are largely preliminary. They suggest, however, that further study of cells from patients with the various types of retinoblastoma may help to elucidate mechanisms for the radiosensitivity of human cells, as well

as the role of DNA repair processes in carcinogenesis and genetic susceptibility to cancer.

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ENVIRONMENTAL CHEMICALS CAUSING CANCER AND GENETIC BIRTH DEFECTS

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Damage to DNA appears to be the cause of most cancer and genetic birth defects and may contribute to aging and heart disease as well. A major part of this DNA damage is caused by environmental chemicals, both natural and man made. Many more chemicals will be added to the current list of human carcinogens and mutagens. Since the late 1950s we have been exposed to a flood of chemicals--from flame retardants in our children's pajamas to pesticides accumulating in our body fat--that were not tested for carcinogenicity or mutagenicity before their use. In the past this problem has been largely ignored; even high-production chemicals, with extensive human exposure, have been produced for decades without adequate carcinogenicity or mutagenicity tests. A few of these chemicals are now being tested in animals, but for most of them the human population is serving as the test animal. Because the 20- to 30-year lag time for chemical carcinogenesis in humans is almost over, the incidence of cancer may increase steeply if too many of the thousands of new chemicals to which humans have been exposed turn out to be powerful mutagens and carcinogens (Figures 1 and 2). We must identify the agents that have caused the cancer and genetic birth defects of today (many of these are natural compounds present in our diet as complex mixtures) and test the many man-made chemicals that have been introduced into the environment in the last few decades. Existing animal tests and human epidemiology *alone* are inadequate procedures for this task for a number of reasons including time, expense, and the difficulty of dealing with complex mixtures.

Over the last 14 years we have developed a simple method for identifying chemical mutagens, and we have shown that almost all chemical carcinogens are mutagens (1). This test combines on a petri plate special strains of *Salmonella* bacteria (as indicators of reverse mutation) and mammalian liver homogenates (rodent or human autopsy--to provide mammalian metabolism) (Figures 3 and 4) (4-6). We have validated the test for detection of carcinogens as mutagens by testing over 300 chemicals and have reported that almost all chemical

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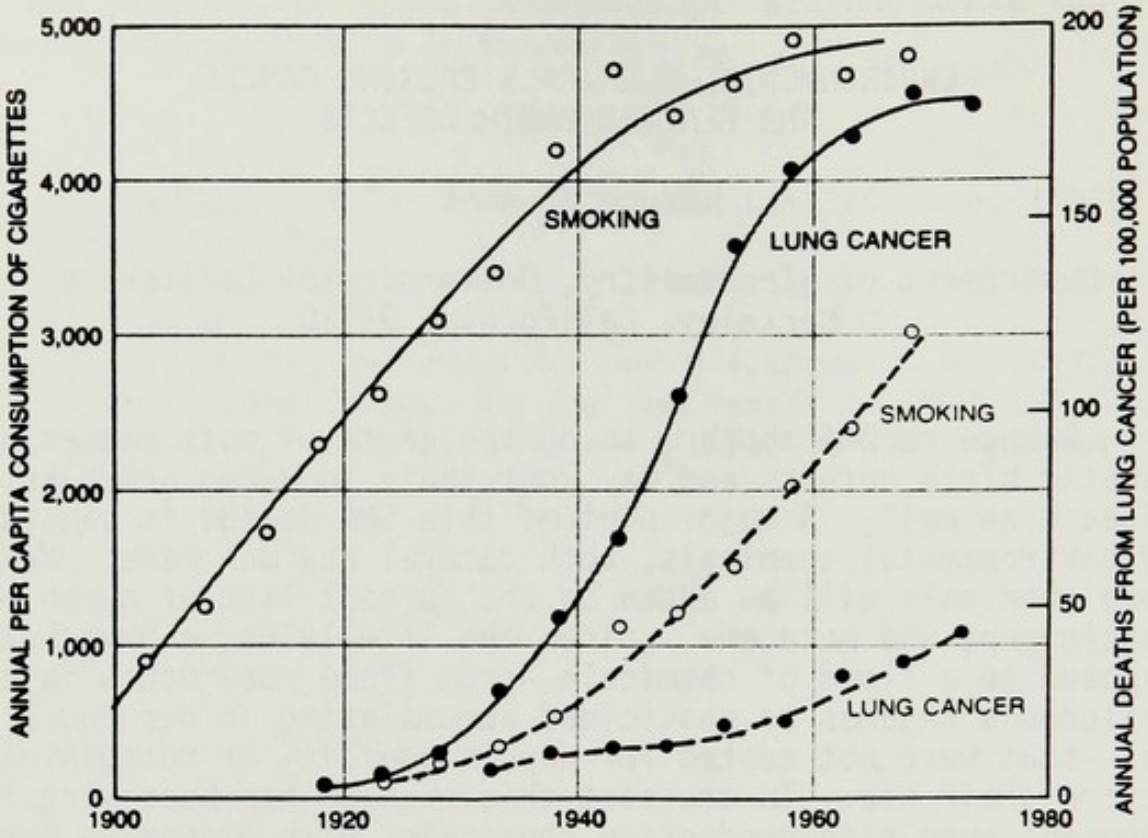

FIGURE 1. Cigarette smoking and lung cancer are unmistakably related, but the nature of the relation remained obscure because of the long latent period between the increase in cigarette consumption and the increase in the incidence of lung cancer. The data are for England and Wales. In men (solid line) smoking began to increase at the beginning of the 20th century, but the corresponding trend in deaths from lung cancer did not begin until after 1920. In women (dotted line) smoking began later, and lung cancers are only now appearing. (From J. Cairns, "The Cancer Problem." Copyright 1975 by Scientific American, Inc. All rights reserved.)

Even if we could identify new hazardous chemicals by human epidemiology, people will already have been exposed for decades, and the discovery may be too late. This happened with vinyl chloride: it was in millions of spray cans and in foods packaged in PVC (polyvinyl chloride) containers. The 20- to 30-year lag time needed for cancer to develop from the exposure to the tremendous increase in chemicals that started during the late 1950s and early 1960s (Figure 2) is almost over. Thus, we may expect a steep increase in human cancer if many of these thousands of new chemicals are indeed powerful mutagens and carcinogens with widespread human exposure.

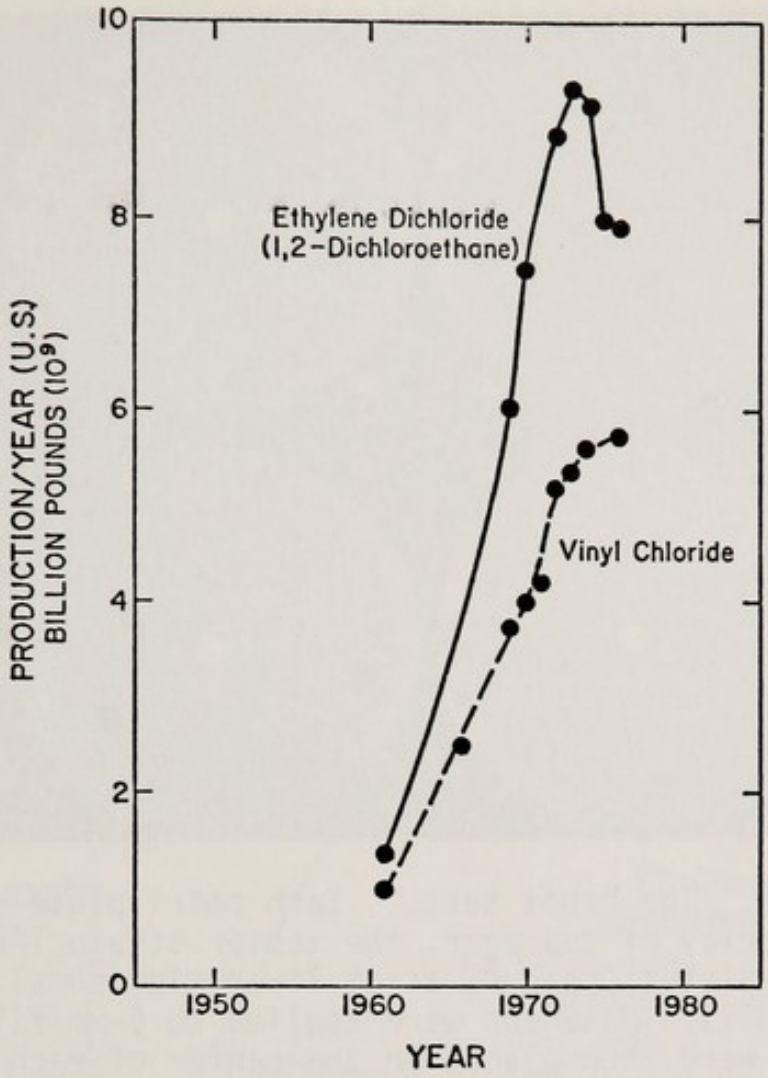

FIGURE 2. Production of two mutagens-carcinogens with widespread human exposure: ethylene dichloride and vinyl chloride (production data from "Top-50 Chemicals" issues of *Chemical and Engineering News*). Approximately 100 billion lb of ethylene dichloride and over 50 billion lb of vinyl chloride have been produced since 1960. Ethylene dichloride is a volatile liquid that is the precursor of vinyl chloride and is also used extensively as a fumigant, solvent, gasoline additive (200 million lb/yr), and metal degreaser. Ethylene dichloride was first shown to be a mutagen in *Drosophila* in 1960, and later in barley and *Salmonella*, but this fact has been ignored. The first adequate cancer test in animals has just been completed by the N.C.I. (November 1977) and is positive in both sexes of both rats and mice. Vinyl chloride gas is used to make polyvinyl chloride (PVC; vinyl) plastic. It was shown to be a carcinogen in rats and in people in the mid-1970s, and a mutagen in *Salmonella* and other systems shortly afterwards. (From B. N. Ames, "Environmental Chemicals Causing Cancer and Genetic Birth Defects," Institute of Governmental Studies, University of California, Berkeley, 1978.)

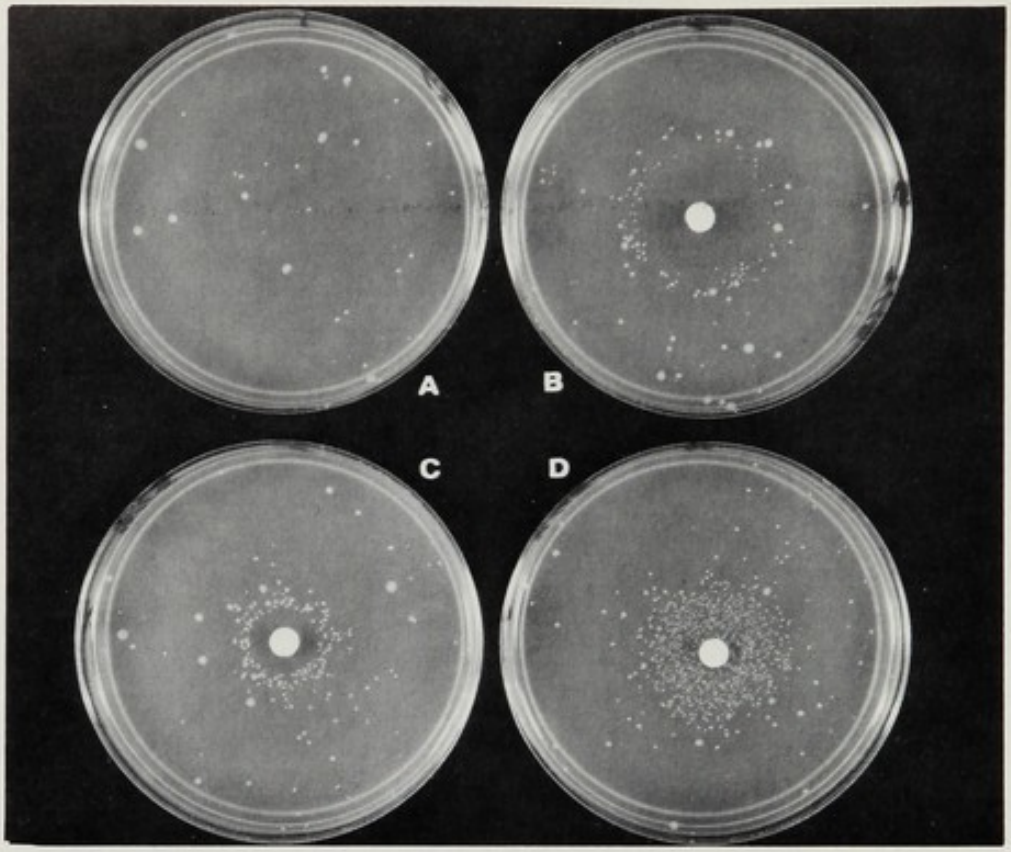

FIGURE 3. The "spot test." Each petri plate contains, in a thin overlay of top agar, the tester strain TA98 and, in the cases of plates *C* and *D*, a rat liver microsomal activation system (S-9 Mix). Mutagens were applied to 6-mm filter-paper discs, which were then placed in the center of each plate: (*A*) spontaneous revertants; (*B*) the Japanese food additive furylfuramide (AF-2) (1 μg); (*C*) the mold carcinogen aflatoxin B_1 (1 μg); (*D*) 2-aminofluorene (10 μg). Mutagen-induced revertants appear as a ring of colonies around each disc. (Reprinted from Ref. 1.)

The food additive furylfuramide was used extensively in Japan from 1965 until recently as an antibacterial additive in a wide variety of common food products such as soybean curd and fish sausage. It showed no carcinogenic activity in tests on rats in 1962 and on mice in 1971. In 1973, Japanese scientists found it to be highly mutagenic in a strain of *Escherichia coli* bacteria (it was also found to be extraordinarily potent in reverting our *Salmonella* tester strain TA100). They subsequently examined it in higher (eukaryotic) organisms and found it to be mutagenic in yeast and *Neurospora*, and to cause chromosome breaks in human white blood cells. Animal tests for carcinogenicity, more extensive than the previous ones, were initiated, and these tests have recently shown that AF-2 is, in fact, a carcinogen. As a consequence of this finding, the Japanese government prohibited the use of AF-2 as a food additive, and all products containing AF-2 were removed from

the market. Since AF-2 had already been tested for carcinogenicity in two animal systems and found negative, it is unlikely that further tests would have been conducted if it had not been shown to be mutagenic.

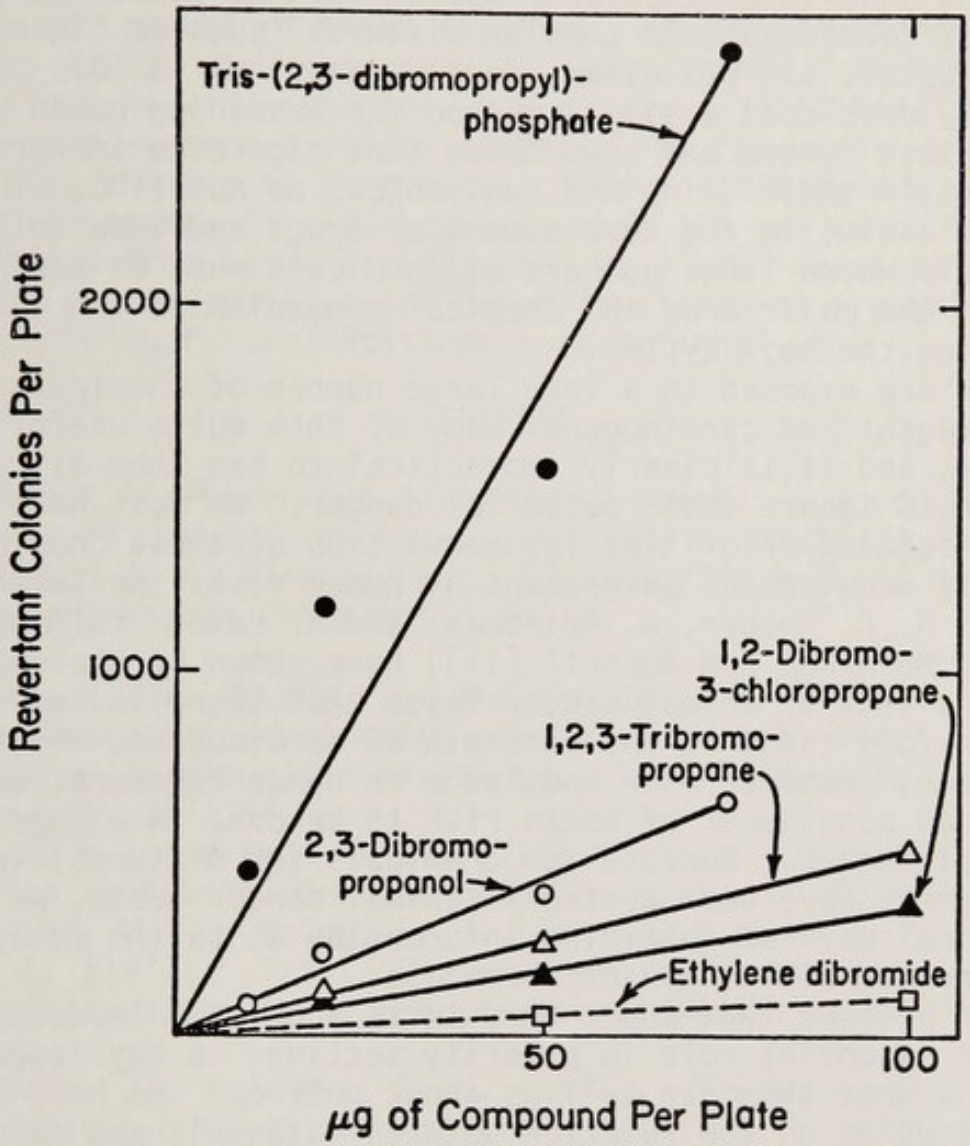

FIGURE 4. The flame retardant *tris*(2,3-dibromopropyl) phosphate and the pesticide dibromochloropropane were in the presence of rat liver homogenate. All compounds were tested on *Salmonella* strain TA100. The amount of the industrial chemical ethylene dibromide added was ten times that indicated on the scale. (Reprinted from Ref. 2.)

Since this and the Prival *et al.* (3) studies, *tris* has been shown to be a carcinogen in rats and mice. Fifty million children have been exposed. Dibromochloropropane (DBCP) has now been shown to have sterilized a large number of factory workers.

carcinogens tested are mutagenic in this test (157/175) and most "non-carcinogens" (95/108) are negative. A number of the "false positives" generated by this study appear to be explainable as consequences of statistical limitations of animal carcinogenicity tests (4,7).

The test is particularly useful for the detection of mutagens/carcinogens in complex mixtures (such as cigarette smoke, water, air pollution, food, and urine) (8-10). We have recently developed a simple method for examining human urine in our test system and have shown that cigarette smokers have mutagens in their urine and non-smokers do not (10). The test is also useful in the development of drugs and industrial chemicals where large numbers of chemicals must be screened. Most of the major drug and chemical companies in the world are now using the test system.

We are exposed to a very large number of chemicals that are mutagens and carcinogens, many of them quite useful for society, and it is clearly impractical to ban them all, yet foolish to ignore their potential danger. We must have some way of setting priorities for regulation of these chemicals, and this requires an assessment of human risk. We (with C. Sawyer, N. K. Hooper, A. Friedman, and R. Peto, following the lead of Meselson and Russell [11]) have shown by the quantitative analysis of animal cancer tests that there is over a million-fold range in the strength of carcinogens, and this knowledge, combined with knowledge on human exposure, may enable an assessment of human risk to be done in a more rational manner. Because few chemicals (or mixtures) in the environment have been tested in animal cancer tests, we need additional ways of obtaining information as to the mutagenic and carcinogenic danger of chemicals.

We believe that short-term tests such as *Salmonella* will play an essential role in priority setting: a key issue, however, is what they can tell us about potency. We have preliminary studies on the relation between mutagenic and carcinogenic potency. There are a number of reasons why one should not expect a very close quantitative correlation between mutagenicity in bacteria and carcinogenicity in animals. Nevertheless, there is over a million-fold range in mutagenic potency in the *Salmonella* test and a similar range in carcinogenic potency and even a rough quantitative correlation would be very useful in human risk assessment. There is an indication, from Meselson and Russell's work (11) and our own that there is a quantitative correlation, not only for carcinogens in the same chemical class, but also across a broad range of classes. Further work will show how general this correlation is. There appears to be a correlation between mutagenic potency in *Salmonella* (and at least one short-term test using human cells [12]). Thus it appears likely that potency in a

battery of short-term tests (many good ones have now been developed) may be able to be used as an aid in human risk assessment.

We believe that a major area of public health can best be attacked by prevention: identifying environmental mutagens/carcinogens, making a rough estimate of human risk based on potency and amount of human exposure, and minimizing human exposure to the more dangerous of these agents. It seems likely that we will soon know how dearly we will have to pay in increased cancer and birth defects for the modern world of industrial chemicals, pesticides, food additives, and plastics. It appears likely, however, that the new methods and discoveries from basic biology that have been developed over the last decade and that are being developed at present will help in making future decisions more rational.

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I have kept the reference list fairly brief. A good general reference is *Origins of Human Cancer*, H. H. Hiatt, J. D. Watson, and J. A. Winsten, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1977).

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WORKSHOP SUMMARY: Chemical Damage and Mutagenesis in Mammalian Systems

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From the diverse contributions of the six speakers at this Workshop three major themes can be resolved. The first concerns the possibility of the existence of different repair modes for different classes of DNA damage in mammalian cells. Both Strauss and Roberts produced biochemical evidence that suggests that cells may have available a number of alternative repair pathways to modify a specific type of damage. Thus it is possible to produce evidence that the excision repair processes which handle the aryalkyl residues may be discriminated from those which modify thymine dimers produced by UV irradiation (Dipple and Roberts, 1977). Although chemical adducts can be excised they may also be bypassed by the replication machinery, the mechanism of bypass probably involves branch migration (Higgins, Kato and Strauss, 1976). The consequences to the cell of the continued existence of adducts is not known but they may be diluted out of the system if replication can continue, in addition they are still available as substrates for excision repair.

Support, at the biological level, for the existence of differential repair modes was provided by Maher and Arlett. Maher showed that defects in excision repair, particularly in xeroderma pigmentosum (XP) cells are very serious for the lethal response when cells are treated with UV or chemicals which produce 'UV-like' distortion in the DNA. Indeed, the more severe the defects in excision the more severe are the effects on cell killing or mutation. Other chemicals which do not produce 'UV-like' damage show no differential killing effects in XP cells. These data indicate a specificity of recognition of the lesion by a particular repair process. These observations were complemented by those of Arlett who showed that by exposing an array of human mutants with specific sensitivities to DNA damaging agents little cross sensitivity was observed. Further, the human DNA damage-sensitive syndromes can be used to identify the mode of action of other agents (Arlett, 1977).

A second major theme was concerned with the complexities of the technology involved in performing mutation studies with cultured mammalian cells. Fox illustrated the difficulties which are encountered in these experiments by showing that the shape of the mutation dose response curves, which are vital for the proper evaluation of the data, are critically dependent upon experimental design. The linear dose-response curves produced by the so-called replating technique (Fox and

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McMillan, 1977) were considered to be the most meaningful. Using this experimental design the absence of an effect of caffeine on either ethyl methanesulphonate or UV-induced mutagenesis in Chinese hamster V79 cells implicates a caffeine insensitive process in mutation which can be contrasted with a caffeine sensitive component to cell lethality. Maher showed how considerable economies can now be made in mutation studies with human cells by using a new technique with bulk culture vessels (Cox et al. 1976). This technique has also been used by Arlett who showed γ radiation mutation results for ataxia telangiectasia (AT) and normal cells. The data are consistent with the concept that the AT cells are hypomutable or at least not more mutable than normal cells, a result which is surprising in the light of the hypermutability of XP cells to UV light (Maher et al. these Proceedings). A possible explanation for this result is that the repair processes which are defective in AT and which convey hypersensitivity to the lethal effects of ionizing radiation (Taylor et al. 1975) are also responsible for the generation of γ-induced mutants in normal cells. This situation has analogies with the effects of the phenotypes of the lexA and recA mutations in E.coli. Here hypersensitivity to UV for cell killing but immutability by it are observed.

The third theme was largely exemplified by the contribution of Trosko who sought to show that somatic cell mutation experiments can be used as a model for carcinogenesis. He showed that tumour promoter agents increased the recovery of UV-induced mutations in Chinese hamster cells while anti-promoters reduced their recovery. Trosko wished to interpret these results to indicate that tumour initiation is the result of some error-prone DNA repair mechanism which leads to a mutation and that tumour promotion is the result of epigenetic processes involving cyclic nucleotide modulation of gene expression. This approach is clearly of great potential in the study of carcinogenesis but these important results need confirmation because of the difficulties, as described by Fox, in performing such experiments.

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BIOLOGICAL CONSEQUENCES OF X-RAY INDUCED DNA DAMAGE AND REPAIR PROCESSES IN RELATION TO CELL KILLING AND CARCINOGENESIS

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ABSTRACT Studies of X-ray transformation *in vitro* suggest that the DNA lesions and repair processes which are primarily responsible for cell killing differ from those which lead to malignant transformation. Lethality appeared to be associated with chromosome aberrations and unrejoined DNA strand breaks, while transformation correlated with the induction of sister chromatid exchanges. It is proposed that *Ataxia telangiectasia* cells may have a defect in the repair of the lethal type of X-ray lesions, whereas hereditary retinoblastoma may involve a defect in the repair of the type of lesions which lead to transformation. The available data suggest that both lethality and transformation induced by UV light and UV-like chemical agents are related to the excision repair of base adducts.

INTRODUCTION

It has recently been shown that most chemical carcinogens are also mutagens. Radiation is mutagenic; X-radiation and ultraviolet light are known to induce specific types of DNA damage, and to induce specific DNA repair processes. Many chemical carcinogens bind to and damage DNA molecules in cells. Exposure to such chemical carcinogens also induces molecular DNA repair processes; in some cases, chemical carcinogens have been classified as "X-ray-like" or "UV-like" depending upon the type of DNA repair activity they induce. These findings have focused attention on the overall role of DNA damage and repair in carcinogenesis. However, direct experimental evidence to relate molecular repair processes to carcinogenesis has been lacking.

I would like in this report to present data which relate to two experimental approaches to the examination of the role of DNA repair processes in carcinogenesis. The first involves the study of cells from individuals who are genetically predisposed to the induction of radiation-induced or spontaneous

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cancer, whereas the second involves the study of the relationship between DNA repair processes and the induction of malignant transformation *in vitro*. The data I will present in both cases relate to DNA damage and repair induced by X-irradiation. Although these data do not as yet establish a specific relationship between DNA repair processes and carcinogenesis, they do suggest possible research directions which may eventually prove useful in establishing such a relationship. They also allow the proposal of certain hypotheses regarding the existence of separate classes of DNA lesions and repair processes for lethal and carcinogenic damage.

GENETIC SUSCEPTIBILITY: RETINOBLASTOMA

Interest in the elucidation of DNA repair defects in cells from individuals who are genetically predisposed to developing cancer was given impetus by the initial observation that the sun sensitive syndrome *Xeroderma pigmentosum* was associated with a DNA repair defect in somatic cells. Other diseases associated with a higher than normal incidence of cancer and which have received recent interest include *Ataxia telangiectasia* (AT), Fanconi's anemia and Bloom's syndrome. These latter diseases, however, are all associated with a variety of perturbations in the immune system (1), and expression of neoplasia is primarily in the hematopoietic or lymphoreticular systems. This factor brings into question the significance of the DNA repair defects identified in some patients with these syndromes in terms of their increased cancer incidence.

We have been studying cells from patients with the malignant eye tumor retinoblastoma (2-4). This disease is associated with no known immunological defect, and both radiation-induced and spontaneous tumors are mesenchymal in origin. It thus appears to be an interesting model to examine the possible role of DNA repair defects in spontaneous and X-ray induced cancer.

Retinoblastoma may appear sporadically or occur in genetically predisposed individuals. The latter group, referred to as the hereditary type, consists of patients who inherit the tumor in an autosomal dominant mode (80-90% penetrance). Hereditary retinoblastoma patients have a significantly increased risk for the development of other tumors both at distant sites and within the irradiated field following radiation therapy. Rarely, retinoblastoma may be associated with a specific deletion in the long arm of chromosome 13 (D-deletion type) which occurs in all somatic cells. Since these patients die early from congenital abnormalities, second tumor formation has not been reported.

TABLE I
X-RAY SENSITIVITY OF RETINOBLASTOMA AND NORMAL HUMAN DIPLOID FIBROBLASTS IN VITRO[a]

Strain	Clinical classification	No. of experiments	D_o±1 S.E.
HL 21	Sporadic retinoblastoma	3	168±11
RBME	" "	3	167± 7
RBCH	" "	3	164± 7
HL 23B	" "	3	144± 7
AG 1947	" "	3	142± 6
AG 1946	" "	3	141± 6
AG 1979	" "	3	135± 8
AG 1980	Hereditary retinoblastoma	3	140± 8
AG 1131^T	" "	3	132± 4
AG 1879^D	" "	5	128± 9
AG 1123^T	" "	3	121± 4
AG 1408	" "	3	119± 5
AG 1880^M	" "	6	98± 6
AG 1142	D-deletion retinoblastoma	6	94± 5
LKR	" "	4	136± 9
Li 106	Normal	8	149± 7
AG 1518	"	4	152±12
GM 1381	"	3	148± 9
EX 25	"	3	140± 4

[a]Data from Weichselbaum, Nove and Little (3).

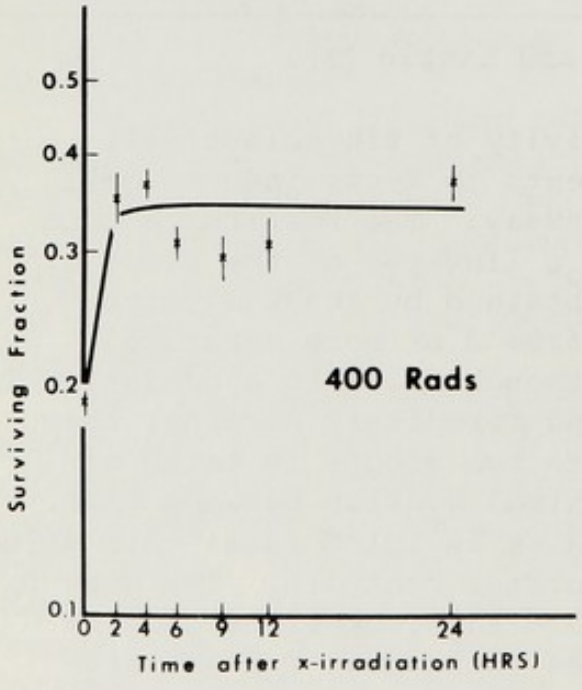
We have examined the sensitivity of fibroblast cell strains from retinoblastoma patients to X-ray induced cell killing by the colony formation assay. The results to date (3) are shown in Table 1. The D_os (inverse of the slope of the X-ray survival curve) were obtained by least squares regression analysis of the data from 3 or more separate experiments for each strain. Although there is a gradation in sensitivity among the sporadic and hereditary strains, they appear to fall very generally into two groups in terms of their X-ray sensitivity, with minimal overlap between them. The mean D_o for the sporadic strains is 151±5 rads; this value is very similar to that for the normal controls. The mean D_o for all hereditary strains is 123±6 rads, or 128±4 rads if AG 1880 is excluded; in both cases these mean D_os are significantly different ($p<0.05$) from the mean D_o for the sporadic strains. None of these strains, however, exhibited the extreme sensitivity (D_o=46 rads) we have found for an AT cell strain (2).

The results in Table I lead us to conclude that fibroblasts from patients with hereditary retinoblastoma are slightly to moderately more sensitive to the lethal effects of X-rays than are normal cells. As a group, sporadic retinoblastoma strains fall well within the normal range.

DNA REPAIR AND MALIGNANT TRANSFORMATION

The second approach we have followed is the study of the relationship between DNA repair processes and malignant transformation in vitro during recovery from potentially lethal damage (PLD) induced by radiation and chemical agents. These experiments have been carried out with confluent (plateau phase) cultures of 10T1/2 mouse embryo fibroblasts, and are similar to liquid holding recovery experiments in bacteria. We initially showed that when subculture of such confluent cultures at low density was delayed for several hours after they were exposed to X-rays, cell survival was significantly enhanced (5). This recovery phenomenon is shown graphically in Figure 1 for 10T1/2 cells exposed to 400 rads. PLD recovery has since been shown to occur after exposure of mammalian cells to ultraviolet light and a number of chemical agents.

We have recently obtained evidence that PLD recovery reflects the action of molecular DNA repair processes (6). XP cells (complementation group A) showed no PLD recovery following UV light exposure, but normal recovery following X-ray



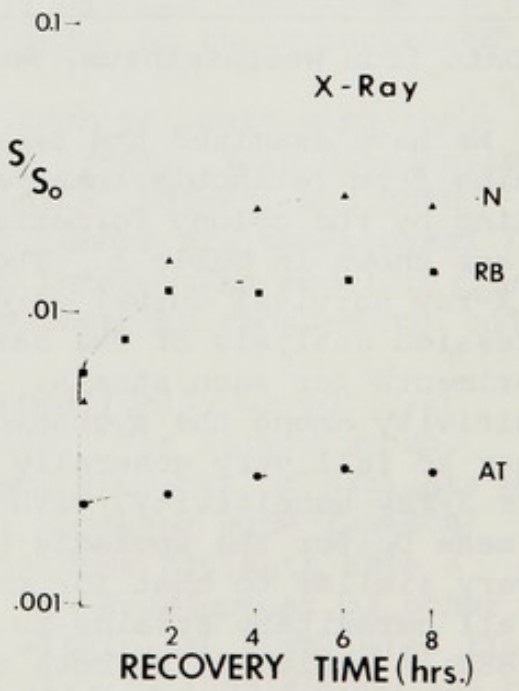


FIGURE 1. PLD recovery in x-irradiated mouse 10T1/2 cells.

FIGURE 2. PLD recovery in normal (Li106), hereditary retinoblastoma (RB), and ataxia telangiectasia (AT) fibroblasts.

exposure. XP variant cells, unlike cells from classical XP, were able to carry out PLD recovery following both X-ray and UV exposure. Rodent cells show a reduced recovery capacity after UV exposure. These results suggest that, as in bacterial cells, PLD recovery does reflect molecular repair--specifically the excision repair pathway for UV-induced damage. A similar conclusion was reached by Simons (7) and by Konze-Thomas et al. (8) who specifically correlated the enhanced survival with dimer excision. PLD recovery following X-ray exposure is shown in Figure 2 for normal (Li106), AT and hereditary retinoblastoma (AG 1880) cell strains. The AT cells are clearly defective in their ability to carry out X-ray induced PLD recovery, but showed normal recovery following UV-irradiation.

The results of experiments are presented in Figure 3 in which density-inhibited plateau phase cultures of 10T1/2 cells were X-irradiated, then returned to the incubator and held in the growth-inhibited state for varying periods of time before subculture at low density to assay for survival or malignant transformation (9). PLD recovery is manifested by an enhancement in survival as is shown in the upper curve (1200 rads). The lower curves represent changes in the transformation frequency during recovery from damage induced by 200, 300 and 400 rads respectively. As can be seen in Figure 3, recovery

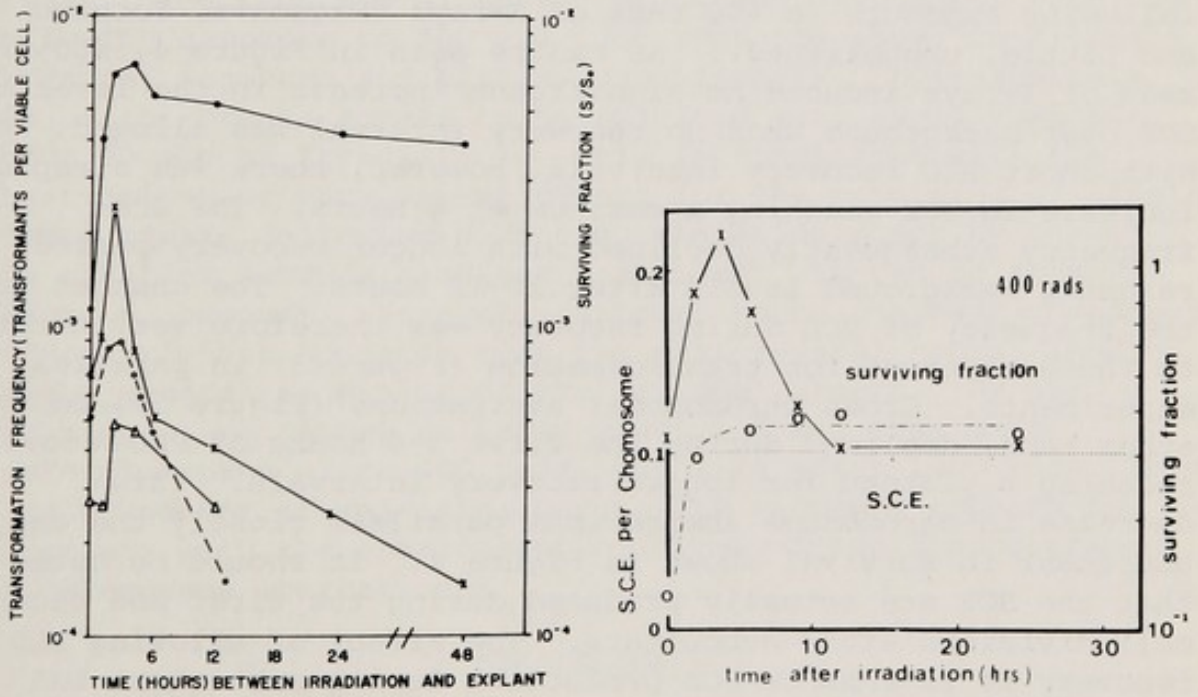


FIGURE 3. Changes in survival (1200 rads, upper curve) and oncogenic transformation (200, 300, 400 rads) during PLD recovery following x-irradiation of 10T1/2 mouse cells (9).

FIGURE 4. Induction of sister chromatid exchanges (SCE) during PLD recovery following x-irradiation. Dotted line is the spontaneous SCE frequency (0.1 per chromosome) (11).

was associated initially with an enhancement in the transformation frequency, reaching a maximum at 3-4 hours, followed by a decline with longer recovery intervals. We have hypothesized (9,10) that these changes in transformation during PLD recovery are the result of the action of DNA repair processes; in particular, we proposed the involvement of an error-prone repair process during the early phase of the recovery period.

In order to further investigate the relationship between processes associated with lethality and survival, similar PLD recovery experiments were performed with 10T1/2 cells and the production of chromosome aberrations and sister chromatid exchanges scored (11). Gross chromosomal aberrations are generally lethal events and have often been associated with double strand breaks. SCE appear to correlate with the presence of long-lived lesions in DNA during replication (12). It has been proposed that SCE may reflect DNA repair processes (13,14) or be a manifestation of mitotic recombination (15). We have studied SCE by a modification of the Perry and Wolff technique (16); the cells were irradiated in confluent growth and were incubated with BrdU beginning immediately after subculture (after irradiation). SCE were scored after two rounds of cell division.

The changes in the frequency of SCE are shown in Figure 4 and of chromosomal aberrations in Figure 5 during PLD recovery following exposure to 400 rads of X-rays (Nagasawa, Fornace and Little, unpublished). As can be seen in Figure 4, 400 rads of X-rays induced no significant increase in the level of SCE over background when no recovery interval was allowed. With short PLD recovery intervals, however, there was a rapid increase in SCE reaching a maximum at 4 hours. The SCE frequency subsequently declined with longer recovery periods reaching background levels after 10-12 hours. The changes in the frequency of SCE during recovery was therefore very similar to those observed for transformation (Figure 3) in parallel experiments. Gross chromosomal aberrations (Figure 5), on the other hand, declined during the first 3-4 hours of PLD recovery reaching a plateau for longer recovery intervals. This decrease in chromosome aberrations parallels closely the enhancement in survival shown in Figure 1. It should be noted that the SCE are actually produced during the first and second cell divisions after subculture. The effect of allowing PLD recovery is to enhance SCE production during subsequent DNA replication.

We are currently trying to correlate these cellular effects with molecular DNA repair processes. We have studied the rejoining of DNA strand breaks by a modification of the alkaline elution technique (17) in which the cells are treated with proteinase-k to remove the DNA crosslinking effect (18) and allow detection of low levels of residual DNA strand

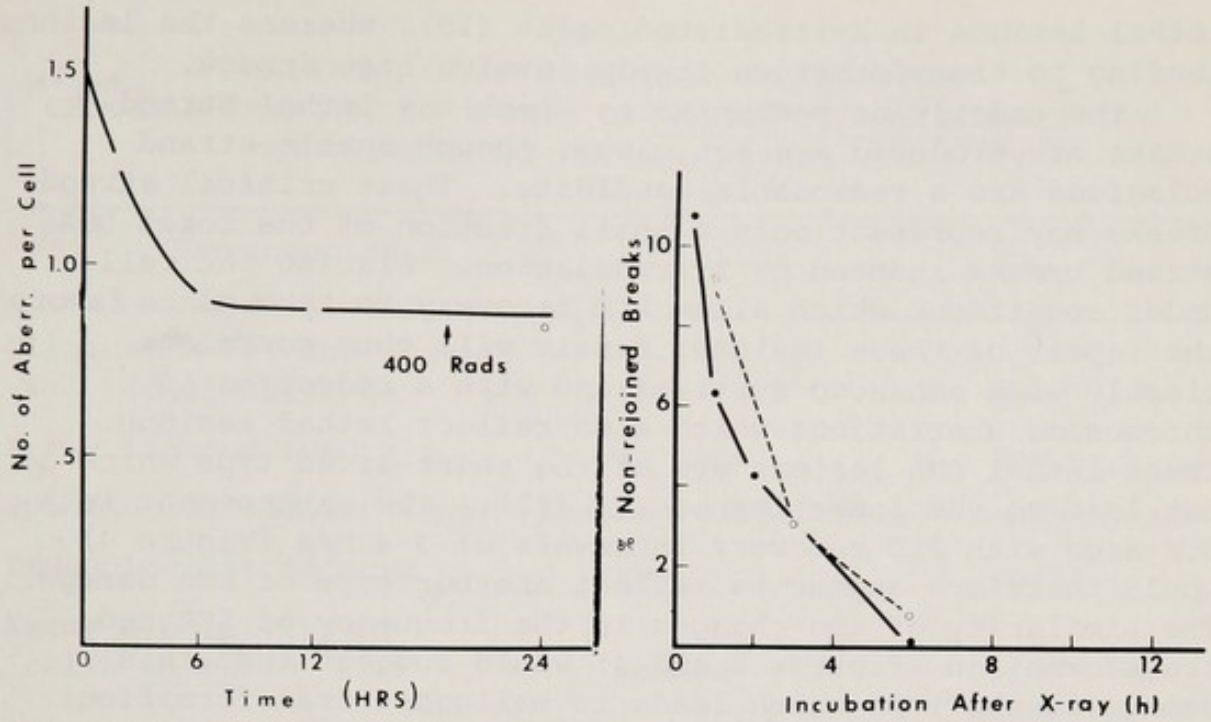


FIGURE 5. Changes in the frequency of chromosome aberrations during PLD recovery.

FIGURE 6. Rejoining of X-ray-induced strand breaks following 400 and 1200 rads during PLD recovery.

breaks. The kinetics of strand rejoining during PLD recovery following exposure to 400 and 1200 rads are shown in Figure 6 (Fornace, Nagasawa and Little, unpublished). Approximately 90% of the breaks appear to be rejoined very rapidly,within about 30 min of irradiation. This is consistent with the observations of many investigators for the rejoining of single strand breaks following much high radiation doses by use of the alkaline sucrose gradient technique. As can be seen in Figure 6, however, there also appears to be a slower component of repair which operates over the next several hours, leading to the gradual rejoining of the residual breaks. By our techniques, we cannot reliably measure fewer than 3-5% residual breaks after 400 rads, and 1-2% after 1200 rads. This slow component of rejoining during PLD recovery appears to correlate with the enhancement of survival and disappearance of chromosome aberrations.

DISCUSSION

Based on these results, I propose the following hypothesis. There are two distinct classes of DNA lesions and repair processes in X-irradiated mammalian cells: those which are primarily responsible for cell killing and those which lead primarily to mutagenesis and transformation. Residual unrejoined or slowly rejoining strand breaks are the primary

lethal lesions in X-irradiated cells (19), whereas the lesions leading to transformation likely involve base damage.

The underlying mechanism by which the lethal strand breaks are produced are not clear, though double strand scissions are a reasonable candidate. These critical strand breaks may represent only a small fraction of the total DNA strand breaks induced by X-irradiation. Placing the cells under conditions which allow PLD recovery to take place favors the repair of these lesions; repair will thus correlate closely with enhanced survival and with a reduction in chromosome aberrations which also reflect lethal lesions. These lethal DNA lesions are of the short-lived type which do not lead to the induction of SCE (12). The enhancement in SCE seen with PLD recovery intervals of 3-4 hrs (Figure 4) would therefore appear to reflect another type of DNA damage. The similarity of the changes in the frequency of SCE and transformation (Figures 3 and 4) would suggest that this damage is the type which leads to malignant transformation.

The DNA lesions which lead to SCE and transformation are likely of the long-lived type such as the base damage induced by UV light exposure. The enhancement in SCE and transformation seen with short recovery intervals would imply an increase in the ratio of transformational to lethal lesions amongst the irradiated cell population. One could envision two mechanisms which might produce this effect. In the first, lethal and transformational lesions would be produced by the initial radiation exposure, but the lethal lesions would be repaired much more rapidly. Thus, with a recovery interval of 3-4 hrs, cells which would otherwise have died would now survive but with long-lived DNA lesions still present. This mechanism would presuppose that the ultimate transformation frequency amongst those surviving cells which recovered from potentially lethal damage (about 50% of the total surviving population following 400 rads) would be considerably higher than that of the remaining cells. We are currently investigating this possibility by measuring the frequency distribution of SCE amongst surviving cells during PLD recovery.

The second mechanism would involve the actual induction of the transformational lesions in the DNA during the repair of the lethal lesions induced by the X-ray exposure (misrepair); maximal repair at 3-4 hrs would thus be associated with maximal transformation. In either case, the transformational lesions (presumably base damage or alterations) would themselves be repaired by a slower acting repair process, perhaps similar to the excision repair of UVL photoproducts, leading to the decline in transformation frequency and SCE production seen with recovery intervals of 12-24 hours (Figures 3 and 4).

How do these results with malignant transformation in

TABLE 2
CLINICAL CHARACTERISTICS AND IN VITRO
CELLULAR RESPONSE TO X-RAYS OF ATAXIA TELANGIECTASIA (AT)
AND HEREDITARY RETINOBLASTOMA (RB)

Characteristic	AT	RB
Clinical X-ray sensitivity (cell killing)	Yes	No
X-ray induced tumors	(?) No	Yes
Spontaneous tumors (type)	lymphoproliferative	mesenchymal
Immunological defects	Yes	No
X-ray sensitivity of cells in vitro	marked	Slight to moderate
PLD repair capacity	reduced	Normal
Mutability by X-rays	reduced	?

vitro relate to the clinical syndromes of enhanced sensitivity to X-irradiation? Here, I propose the hypothesis that Ataxia telangiectasia (AT) cells are defective specifically in a DNA repair process which acts on lethal lesions, presumably residual strand breaks, whereas hereditary retinoblastoma (RB) is associated with a repair defect for transformational lesions presumably base damage.

Several characteristics of the response of AT and RB patients and their cells to X-irradiation are tabulated in Table 2. The clinical radiosensitivity of AT patients appears related to cell killing; there have been no reports of an increased incidence of X-ray induced cancers in these patients. This clinical sensitivity is associated with an extreme sensitivity of AT cells in vitro to cell killing by X-rays and a much reduced capacity for PLD recovery (Figure 2), but a reduced sensitivity to the induction of mutations (20). These findings are thus consistent with the hypothesis proposed and discussed by Lehmann (19) that unrejoined strand breaks are the cause of cell death after X-irradiation, and that the defect in AT involves the repair of some class of DNA strand breaks. The increased incidence of lymphoproliferative tumors in AT patients is most likely related to the immunological perturbations associated with this disease rather than the repair defect.

In RB, on the other hand, the clinical X-ray response involves the induction of cancer in the irradiated field;there

is no evidence for an enhanced sensitivity to cell killing *in vivo*. Unlike in AT, RB patients have no reported immunological defects, and the spontaneous and X-ray induced tumors are mesenchymal in origin. Again, unlike AT, RB cells are only slightly more sensitive than normal to killing by X-rays *in vitro* (Table I), and preliminary experiments show a normal or near normal PLD recovery capacity (Figure 2). We are currently examining their sensitivity to the induction of mutations by X-rays. These findings lead us to conclude that if a DNA repair defect is associated with hereditary retinoblastoma, it is related to the repair of lesions which lead primarily to mutagenesis and transformation rather than to cell killing.

The data presented in this paper all relate to the role of DNA damage and repair in the induction of cancer by X-rays. There is also recent evidence to relate the DNA damage and repair processes induced by UV light and UV-like chemical agents to carcinogenesis. Hart *et al*. (21) have presented evidence that pyrimidine dimers induced by UVL in thyroid tissue of a certain species of fish can give rise to tumors. Removal of the dimers by photoreactivation markedly decreased the tumor yield. Ikenago and Kakunaga (22) studied the induction of malignant transformation by 4-NQO in confluent cultures of a strain of mouse 3T3 cells subcultured at various times after exposure to the carcinogen. They found a direct relationship between the excision of 4-NQO adducts and a reduction in the transformation frequency; post-treatment incubation with caffeine greatly reduced the transformation frequency. These findings are of interest in the light of the observations of Maher *et al*. (23) that an enhancement in survival and a decline in mutagenesis in UV-irradiated human cells correlated closely with the excision-repair of pyrimidine dimers. They suggest, furthermore, that the same class of DNA lesions is responsible for cell killing and transformation in UV-irradiated cells.

Finally, Summers and Das Gupta (24) and Lytle (unpublished) have presented evidence that UV-enhanced reactivation of irradiated Herpes virus by mammalian cells is associated both with enhanced survival and enhanced mutagenesis in the virus. This finding offers experimental support for the hypothesis that inducible error prone repair processes exist in mammalian cells. Such a process could explain the findings in Figures 3 and 4, and might be of major importance in the induction of cancer by physical and chemical agents.

In conclusion, the data reported in this paper do not as yet directly relate DNA repair processes to carcinogenesis. I hope, however, that they indicate some research directions which might prove useful in establishing such a relationship.

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CHEMICAL MODIFICATION OF RADIATION-INDUCED MUTAGENESIS AND ITS ROLE IN CARCINOGENESIS[1]

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ABSTRACT The role of DNA damage in mutation fixation and carcinogenesis is well documented. Theoretically, many non-mutagenic chemicals, known to modify carcinogenesis, can modify either the amount of DNA damage, the repair of the damage, or the expression of the genetic consequences of error-prone DNA repair/replication. Various non-mutagenic, tumor-modifying chemicals were tested for their effects on UV-induced ouabain-resistant mutations in Chinese hamster cells. Those tested were shown to modify the frequency of mutations by affecting DNA repair processes or the expression of induced mutations. The results also support the hypothesis that tumor initiation is due to a mutagenic event, while tumor promotion (or anti-promotion) is the result of an epigenetic process.

INTRODUCTION

If DNA damage and its "error-prone" repair play a role in mutagenesis and if mutagenesis plays a role in some forms of carcinogenesis (1), then certain chemicals, which can (a) influence the amount of initial DNA damage; (b) inhibit the repair of that DNA; and (c) modulate the genetic expression of the "error-prone" repair of the damage, ought to be able to modify tumorigenesis. Furthermore, various non-mutagenic chemicals, which are known to modify carcinogenesis, would, in theory, act on one or more of the aforesaid levels.

The two stage ("initiation" and "promotion") theory of carcinogenesis has been demonstrated in many animal systems with either physical or chemical carcinogens as initiators (2,3,4). The two-staged phenomenon has also been demonstrated

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in several in vitro systems (5,6,7), designed to measure transformation and mutagenesis in physical and chemical carcinogen-exposed cells which were posttreated with the tumor promoter, 12-0-tetradecanoyl-phorbol-13-acetate (TPA). If mutagenesis is an important biological process leading to carcinogenesis (i.e., initiation), then one would predict from this 2-stage theory of carcinogenesis that those factors that influence the 2-stage process of tumorigenesis also will influence the frequency of mutations.

Indeed there have been many reports of non-mutagenic chemicals that can modify tumorigenesis. It has been shown that cyclic AMP (8), steroid hormones (9) caffeine or theophylline (10,11), phorbol esters (2), protease inhibitors (12), retinoic acid (13), butylated hydroxytoluene (14), phenobarbital and other compounds (3) ameliorate or enhance the frequency of tumors induced by various physical and chemical carcinogens. Moreover, several of these non-mutagenic chemicals, which have either promotion or "anti-promotion" activities, can negate each other's effect either in the in vivo mouse skin assay or in various in vitro systems designed to measure several biochemical markers associated with tumor promotion. The induction of plasminogen activator (15) and ornithine decarboxylase (16) appears after the treatment of cells with tumor promoters. For example, dexamethasone and fluocinolone acetonide (anti-inflammatory hormones) are potent inhibitors of TPA promotion of 7, 12-dimethylbenz (a) anthracene [DMBA]-induced mouse skin tumorigenesis (9,17), as well as inhibitors of TPA-induced plasminogen activator (17,18). Vitamin A (and its synthetic analogs) can also inhibit TPA-induced ornithine decarboxylase activity in mouse epidermis (19). Cyclic AMP phosphodiesterase inhibitors (caffeine, theophylline) increase cyclic AMP levels and depress the TPA-production of plasminogen activator (20). A 17-β estradiol-induction of vaginal epithelial proliferation can be prevented by treatment with vitamin A (21).

RESULTS AND DISCUSSION

Based on the working hypothesis that initiation is due to mutagenesis and that promotion (or anti-promotion) is the result of gene modulation (derepression or repression of genes), we tested a number of chemicals mentioned above. Using a Chinese hamster (V79) mutation assay system, with either 6-thioguanine-or ouabain-resistance as markers, we designed a protocol to test if these aforesaid chemicals acted as mutagens or gene modulators (see (22) for detailed protocols). Various non-mutagenic chemicals, most of them

known modifiers of carcinogenesis, were tested for their effects on UV-induced 6-thioguanine or ouabain-resistant mutations. They were shown to modify either the mutation-fixation process (i.e., the DNA repair process) or the expression of induced mutations.

Treatment with caffeine or harmane immediately following UV irradiation significantly reduced the colony-forming ability. The mutation frequency was increased with caffeine treatment (23) but was reduced with harmane (24). Fractionation of UV-irradiation always increased colony-forming ability, but reduced the mutation frequencies (25). Treatment with cycloheximide between fractionated UV-irradiations resulted in two types of effects (25). Long exposure to cycloheximide (i.e., > 6 hr) always resulted in reduced survival and enhanced mutation frequency. Exposure to cycloheximide in the short fractionation regime (i.e.,>4 hr) tends to give the opposite effects. The results suggest the presence of error-free and error-prone post replication repair mechanisms in Chinese hamster cells.

TPA, a powerful tumor promoter, increases the recovery of UV-induced 6-thioguanine and ouabain-resistant mutations (7). It can also enhance the frequency of X-ray induced 6-thioguanine resistance (26) and the frequency of methylazoxymethanol acetate-induced ouabain resistant mutations (27). Anti-promoters, such as fluocinolone acetonide, dexamethasone, retinoic acid, caffeine and dibutyryl c-AMP, on the other hand, reduced the recovery of UV-induced oua^r mutants when present at the mutation expression time. These results suggest to us that many induced mutations can either be repressed or derepressed by agents which can alter cyclic AMP levels (22). Furthermore, the results also support the hypothesis that tumor initiation is the result of some error-prone DNA repair mechanism which leads to a mutation and that tumor promotion is the result of an epigenetic process involving cyclic nucleotide modulation of gene expression (1,22).

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BIOLOGICAL AND BIOCHEMICAL EVIDENCE THAT DNA REPAIR PROCESSES IN NORMAL HUMAN CELLS ACT TO REDUCE THE LETHAL AND MUTAGENIC EFFECTS OF EXPOSURE TO CARCINOGENS[1]

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ABSTRACT As part of on-going investigations into the mechanisms of action of physical and chemical carcinogenic agents in cultured human cells, we are examining the effect of cellular DNA repair processes on the cytotoxic, mutagenic and transforming action of such agents. It is well-known that skin fibroblasts derived from normal individuals excise ultraviolet radiation (UV)-induced DNA lesions at a much faster rate than cells derived from xeroderma pigmentosum (XP) patients. Our studies comparing normal and XP cells demonstrate that excision repair processes act to reduce the potentially lethal and mutagenic consequences of UV exposure. We have extended these investigations to a series of chemical carcinogens including reactive derivatives of a number of aromatic amides and polycyclic aromatic hydrocarbons. We find that the survival of human cells exposed to such agents reflects their capacity to repair UV-induced damage to DNA. Similarily, we find that the frequency of mutations to azaguanine resistance induced by these agents in the human cells reflects their capacity for excision repair of UV-induced damage. Results of biochemical studies suggest that XP cells are also abnormally deficient in their ability to remove covalently-bound carcinogen-DNA adducts. Our studies comparing the effects of a series of polycyclic hydrocarbon derivatives in normal human cells indicate that although these agents differ significantly in their cytotoxic and mutagenic effect as a function of concentration administered and produce different DNA-carcinogen adducts, the frequencies of mutations induced by each are comparable when analyzed as a function of the cytotoxic effect.

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This finding suggests that the cellular processes ultimately responsible for the cytotoxic and mutagenic consequences handle the critical lesions in a comparable manner.

INTRODUCTION

During the past decade since the earliest report of the difference between the DNA repair capacity of cells from normal individuals and those from XP patients (1), impressive efforts have been devoted to biochemical and biophysical measurements of excision and "post-replication" repair in mammalian cells. (cf. refs. 2-5 for reviews.) The question we have concentrated on is what are the *biological consequences* of these excision and post-replication repair processes? For this purpose we have compared the cytotoxic and mutagenic effects of a number of DNA-damaging agents in diploid human skin fibroblasts derived from normal individuals, XP patients from various complementation groups, and XP variants. It is clear that many detailed studies are still necessary before we can accurately correlate the biological consequences observed with biochemical measurements of the rate of excision of dimers or removal of particular carcinogen-DNA bound adducts. However, we here summarize the results of some of the comparative studies.

MATERIALS AND METHODS

The procedure we have used to determine the cytotoxic and mutagenic effect of exposure of human cells to UV radiation or to these chemicals have been published (6-11). Procedures developed for reliable use of bulk culture vessels and thioguanine as a selective agent with human cells are similar to those of Cox and Masson (12) but include our own adaptations. Details have been submitted for publication. The technique we use to measure the number of thymine dimers remaining in DNA has been published (13). We have used the method of Lieberman and Dipple (14) to measure the carcinogen residues remaining covalently bound to DNA and bromodeoxyuridine-thymidine H^3 labeling of DNA and alkaline CsCl gradient fractionation to measure the amount of repair replication occurring in cells and the degree of inhibition of semiconservative DNA synthesis following exposure to various concentrations of reactive forms of chemical carcinogens (15).

RESULTS AND DISCUSSION

Low doses of UV radiation sharply reduce the survival of XP12BE cells which are totally incapable of carrying out excision of pyrimidine dimers but do not kill normally excising cells. Cells from patient XP2BE which repair at a rate 16% of normal are somewhat more resistant then XP12BE cells to the killing action of UV. When we compared these three strains for the frequency of mutations to azaguanine resistance induced by UV, a directly corresponding pattern was observed (see Fig. 2, in the paper by McCormick and Maher in this volume and ref. 10). Irradiation with low doses of UV induced a high frequency of mutations in the excision deficient XP strains, but was completely ineffective for inducing mutations in the excision proficient normal human cells. Only at much higher (cytotoxic) doses was the frequency of azaguanine resistant colonies increased above background in a dose-dependent manner. When the frequency of mutations induced in these strains is analyzed as a function of the cytotoxic effect, it is found to be approximately the same for all. This suggests that they handle lesions remaining unexcised in their DNA in a comparable manner.

We have evidence that a similar relationship exists in these normal and XP strains between the capacity for excision repair of UV-induced DNA damage and the mutagenic and/or cytotoxic effect of exposure to certain chemical agents. Examples of such chemicals are 4-nitroquinoline-1-oxide, the N-acetoxy derivative of 2-acetylaminofluorene, 2-acetylaminophenanthrene, 4-acetylaminobiphenyl, 4-acetylaminostilbene, the "K-region" epoxides of benzo(a)pyrene (B(a)P), benz(a)anthracene, dibenz(a)anthracene, 7,12-dimethylbenz(a)anthracene, 7-bromomethylbenz(a)anthracene and 7-bromomethyl-12-methylbenz(a)anthracene and two isomers of B(a)P 7,8-diol-9,10-oxide. Each of these agents caused a greater cytotoxic effect in XP cells than in normal cells and what is more significant, the slopes of the survival curves reflected the repair capacity of the particular strains tested. No such differential was observed with N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-N'-nitrosourea, or methyl methanesulfonate (See Fig. 1). We found that each agent which caused differential survival also produced sites in DNA susceptible to the action of single-strand specific endonuclease S_1, i.e., localized distortions in the helix (16). This suggests that some form of distortion in DNA is the damage recognized by excision processes.

The mutagenic action of a number of reactive metabolites of polycyclic hydrocarbons was also compared. We found that the frequency of mutations induced by a given exposure was

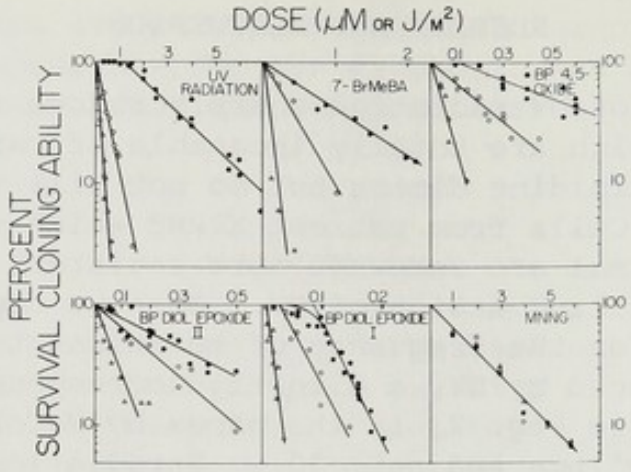

Fig 1. Comparison of the cytotoxic effect of reactive derivatives of carcinogenic agents in diploid human cells. (●) normal; (O) XP2BE (complementation group C); (Δ) XP12BE (group A). Taken from ref. 16.

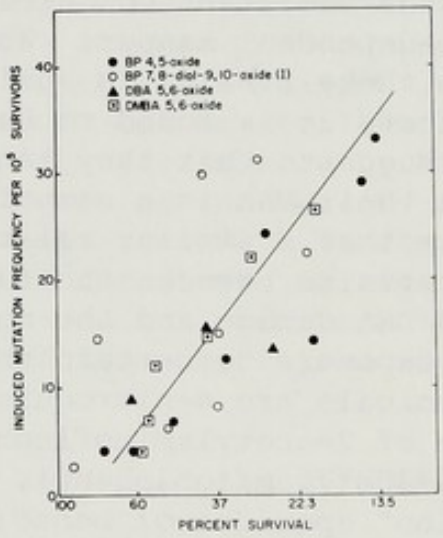

Fig 2. Frequency of mutations to azaguanine resistance induced in normal cells by four hydrocarbon metabolites, plotted as a function of their cytotoxic effect.

higher in XP cells than in normal (11) suggesting that repair reduced some of the potential mutagenic effects of the exposure. Significantly, when the frequency of mutations induced in normal and in XP cells by a particular compound was analyzed as a function of the cytotoxic effect, these were comparable (11). It is clear from their structures and from biochemical binding studies (17-19) and from our own findings with the S_1 nuclease (16) that these hydrocarbon metabolites form different DNA-carcinogen adducts in the cell. Nevertheless, when we compared the frequency of mutations induced in normal cells by four different hydrocarbon derivatives at concentrations which caused equal cytotoxicity, each metabolite proved equally mutagenic (Fig. 2). The results of

these studies suggest that the cellular processes ultimately responsible for the cytotoxicity and mutagenicity of these agents handle them in a comparable manner. We attributed this to the fact that all of them formed a common lesion on the DNA viz., a distorition in the helix (11) which was handled by a common mechanism. Another hypothesis which could explain this phenomenon has been suggested by Roberts (20) and is discussed in this volume by McCormick and Maher.

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EVIDENCE FOR THE INVOLVEMENT OF DIFFERENT REPAIR MECHANISMS IN MUTAGENESIS AND CELL KILLING IN V79 CELLS

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ABSTRACT When selective agents were added to single cells, induction of $6TG^r$ and $8AZ^r$ mutants by EMS and UV in V79 cells was linear with dose. Frequency of $6TG^r$ mutants was lower than that of $8AZ^r$ mutants. Mutation induction, in contrast to cell killing and chromosome aberration induction was independant of DNA replication and was caffeine insensitive. Several problems of methodology have been identified.

INTRODUCTION

Dose response curves obtained by the two methods used for quantitation of induced mutation in mammalian cells differ. A curvilinear response is obtained when *in situ* selection is used[1-5], a linear one on replating[6-8]. In addition, although many purine analogue resistant lines have now been shown to be structural gene mutants[9,10] evidence has recently been presented indicating that $8AZ^r$ lines can arise by other mechanisms[11,12].

In the light of such difficulties, we felt that the reported relationship between chromosome damage, cell killing and mutation induction, and the apparent enhancing effect of caffeine on all three processes needed reappraisal[1-4].

We now report experiments which indicate that there may be a significant contribution to the overall frequency of $8AZ^r$ colonies from non-mutants on *in situ* selection. In addition, we confirm our previous observations[6] of a lack of effect of caffeine on replated mutant frequencies and explain the apparent *in situ* enhancement by caffeine.

RESULTS

FORWARD MUTATION TO $8AZ^r$ OR $6TG^r$

V79 cells were mutagenised with EMS or UV 4 hrs after

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plating (cell multiplicity 1.12). At appropriate times, mutants were selected with 8AZ or 6TG either in situ or after replating. The observed mutant frequencies presented in Table I. show that 1) in situ frequencies exceed replated frequencies: the discrepancy increases with increasing dose for both mutagens. 2) IMF are lower after 6TG selection than after 8AZ. 3) At equitoxic doses EMS is more effective than U.V.

We suspected that the curvilinear in situ response may be partially due to a density dependant loss of mutants before complete expression. The data in Fig.1. support this view since higher in situ frequencies at low doses and consequently a more linear dose response, was obtained when larger plates were used.

As reported by others[7,11], the expression time of mutants was independant of dose but was later in 6TG than 8AZ; in both cases a stable plateau was obtained (Fig.2).

TABLE I

Selective Conditions	Mutagen	Dose or Conc	S.F.[a]	Mutation Frequency x 10^{-4} ± S.E.	
				in situ/ survivor (9cm plates)[b]	replated/ viable cell[c]
30µg/ml 8AZ	U.V. J/m^2	0	1.0	0.64±0.07	0.2±0.05
		50	0.97	1.34±0.2	0.96±0.1
		100	0.67	7.5	2.1±0.2
		150	0.30	11.4±3.6	2.7±0.5
		200	0.08	12.1±1.1	2.8±0.2
	EMS mg/ml (3hrs)	0	1.0	0.66±0.24	0.33±0.03
		0.5	0.9	9.6±3.1	6.6±0.4
		1.0	0.66	18.0±2.2	9.2±0.7
		1.5	0.43	25.0±4.5	11.5±1.2
		20	0.08	101±8.9	16.6±0.9
10µg/ml 6TG	U.V.	100	0.49	6.18±0.6	1.26±0.04
		200	0.27	7.13±0.4	1.37±0.24

a) S.F. was always determined on same mutagenised population
b) Frequencies calculated from at least 2-3 plateau values
c) Frequencies calculated from 4-10 separate determinations 66-350 hrs after treatment (Fig.2).

EFFECT OF CAFFEINE ON FORWARD MUTANT FREQUENCY

Potentiation of mutagen induced chromosome damage and

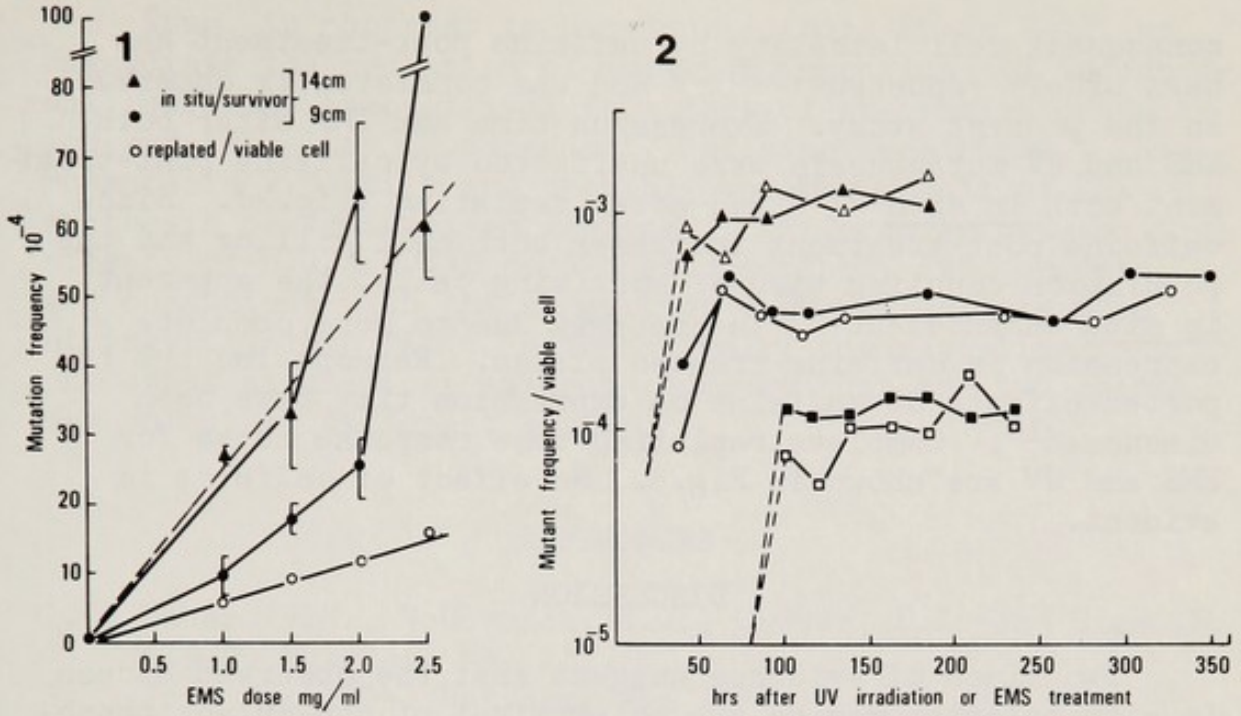

FIGURE 1. Dose response curves for EMS induced $8AZ^r$ colonies determined by different methods. ---- Indicates the probably linear response when 14cm plates are used. Selection in 30µg/ml 8AZ.

FIGURE 2. Replating expression curves for EMS and UV induced mutants with (open symbols) or without (closed symbols) 24hrs post treatment exposure to 0.75mM caffeine △ ▲ EMS 1mg/ml 3 hrs 8AZ 30µg/ml ○ ● 15.0J/m^2 8AZ 30µg/ml □ ■ 10.0J/m^2 6TG 10µg/ml.

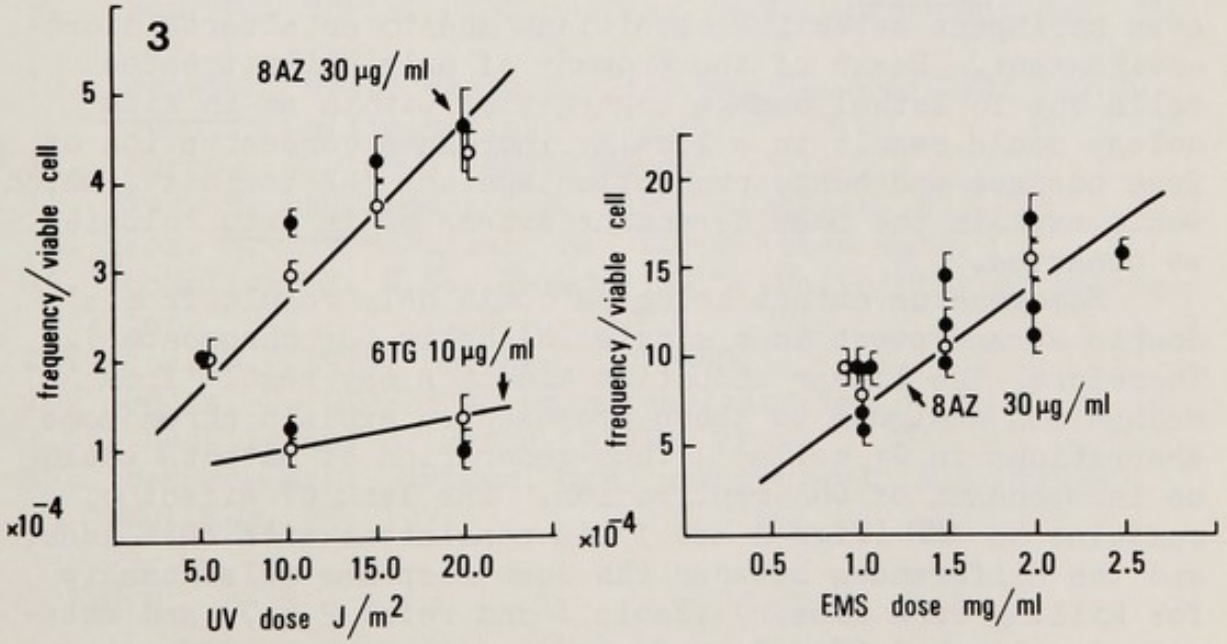

FIGURE 3. Replated dose response curve for UV and EMS induced mutation to $8AZ^r$ or $6TG^r$ ● no caffeine ⊙ 0.75mM caffeine 24 hrs post treatment. Points are mean ± S.E. of 4 - 10 separate determinations.

consequent cell lethality by caffeine post-treatment has been widely reported[2,3,6,16] and was consistently observed in the present study. Expression time and IMF after both EMS and UV mutagenesis were unaffected by caffeine post-treatment both *in situ*[13,14] and after replating (Fig.2). Since caffeine post-treatment increases both cell killing and the population doubling time of surviving cells, the apparent *in situ* enhancement[2-4] is probably due to more complete expression in caffeine treated plates. Reasons for the reported effects of caffeine on expression time have been discussed[14]. Complete replating dose response curve for EMS and UV are shown in Fig.3. No effect of caffeine is evident.

DISCUSSION

Two lines of evidence suggest that the observed excess *in situ* colonies may be due to survival of non-mutant phenotypes. Firstly, we have observed an inverse relationship between killing and cell density at the time of treatment[13].

Secondly, an excess of replated colonies would be expected if there were significant intra-colony heterogeneity, and we have observed a replating increment when treatment multiplicity was >1[15]. Thus we assume that metabolic co-operation functions in these cells which leads us to conclude that the majority of mutants arising after treatment at single multiplicity (Table I, Fig.1.) must be in homogeneous colonies. Therefore, *in situ* colonies may be non-mutants which survive even stringent selective conditions due to an altered microenvironment. Death of the progeny of originally treated cells due to lethal damage segregation within an *in situ* colony could result in a locally increased concentration of free purines and hence protection against 8AZ toxicity, which would explain the dose dependant excess of *in situ* colonies we observed.

Homogeneous mutant colonies could only result from a double strand event in a single DNA helix (G_1 chromosome). Therefore, the linear induction kinetics may result from mechanisms analgous to those proposed to explain chromosome aberrations in G_1 cells[17], thus generation of mutants would be independant of DNA replication. The lack of effect of caffeine on IMF (Figs 2 and 3) is consistent with this idea, and the differences between the dose response relationship for killing (non linear) (Table I and refs. 2,6,7) and mutagenesis (linear) (Fig.3. refs 6,7) suggest that different lesions or different repair processes are involved. In contrast, the induction of chromosome aberrations and associated cell lethality and the amplification of such effects by caffeine is dependant on DNA replication[3,17,17].

Thus, in contrast to previous authors (refs 1-4), we dissociate the mechanism of mutation induction (S independant, caffeine insensitive) from that of aberration production and lethality (S dependant, caffeine sensitive). Thus mutants in V79 cells are probably not the result of DNA synthesis over unexcised lesions[6]. Differences between the effects of 6TG and 8AZ have been described, and our data are consistent with the suggestion that 6TG selects more stringently than 8AZ[11], alternatively, the replating data and other biochemical evidence suggest that the two agents may select at different loci.

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EFFECTS OF LIQUID HOLDING ON CELL KILLING AND MUTATION INDUCTION IN NORMAL AND REPAIR - DEFICIENT HUMAN CELL STRAINS

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ABSTRACT Liquid holding conditions can be obtained for human diploid fibroblasts by holding confluent cultures stationary for seven days by means of conditioned medium. Under this condition recovery of radiation damage induced by ultraviolet light or X-rays is observed as an increase in cloning efficiency. XP25Ro and XP7Be (Xeroderma pigm., compl. gr. A and D) exhibit less recovery after UV-irradiation than wild type and the same was observed for AT5Bi (Ataxia tel.) after X-irradiation. During liquid holding of UV-irradiated cells the mutant frequency was considerably reduced in XP7Be cells, while in wild type cells the mutant frequencies were the same or lower at the lower exposures and the same or higher at the higher exposures.

INTRODUCTION

Increased viability of E. coli B cells irradiated with UV is found if after irradiation, the bacteria are held in the dark in liquid suspension in saline (1). This phenomenon became known as "liquid holding recovery" (LHR). It is generally assumed that under liquid holding (LH) conditions DNA-repair occurs exclusively through excision repair while replication and thus post replication repair does not take place (2).

For mutagenized mammalian cells recovery of potential lethal damage was demonstrated in density-inhibited cultures of Chinese hamster cells (3). During density inhibition most

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of the cells are in an extended G_1. However the cells are not completely blocked and cell killing is often not negligible (4, 5). Therefore, in this study we used human diploid skin fibroblasts which, when confluent, are characterized by a strong degree of contact inhibition of cell division.

MATERIALS AND METHODS

Wild type cell strains were derived from normal foreskins. XP7Be was obtained from the American Type Culture Collection. XP25Ro and AT5Bi were gifts from Dr. D. Bootsma, Rotterdam, and from Dr. C. Arlett, Sussex, respectively.

Ham's F-10, modified by the omission of hypoxanthine and supplemented with 15% newborn calf serum was used as standard medium. Medium with 5 µg 6-thioguanine per ml was used for the selection of HGPRT-deficient mutants.

The UV-irradiations were carried out on attached cells at an exposure rate of 0.9 or 0.1 J/m^2/sec. X-irradiation was performed on cells in suspension at an exposure rate of 395 R/min (150 kV, 6 mA). For expression of induced mutants the cells were cultured over a period of 7 or 8 days. Details of the mutational assay system have been described earlier (6).

RESULTS

Condition of Liquid Holding. Cultures with cells in a stationary phase were obtained by seeding 1 x 10^6 cells per 90 mm petri dish. When the cultures reached confluency after one or two medium changes, they received a final medium change with 15 ml of medium; the fifth day after this last medium change was taken as the starting-point for the liquid holding period. The cell number showed no obvious changes during the first 2 days. Over 7 days a small decrease or increase in cell number could be observed. Cell multiplication was not compensating for cell loss as the frequency of cells in S-phase after continuous labeling during 7 days LH amounted to only 10% in 2 experiments and were almost absent in 2 other experiments. Even a LH-period of 21 days appeared possible.

Effect of LH on Cell Killing and Mutation Induction. The effect of LH over 1 to 7 days on the survival of wild type (WT) and XP7Be is shown in figure 1. Recovery in survival appears to take place over a long time in the case of XP7Be. The effect of 7 days LH on survival was determined in UV-irradiated WT, XP25Ro and XP7Be cells and in X-ray-irradiated WT and AT5Bi cells. The degree of LHR was quantitated by comparison of the slopes of the survival curves, which results in a dose-modifying-factor (DMF). After UV-irradiation the

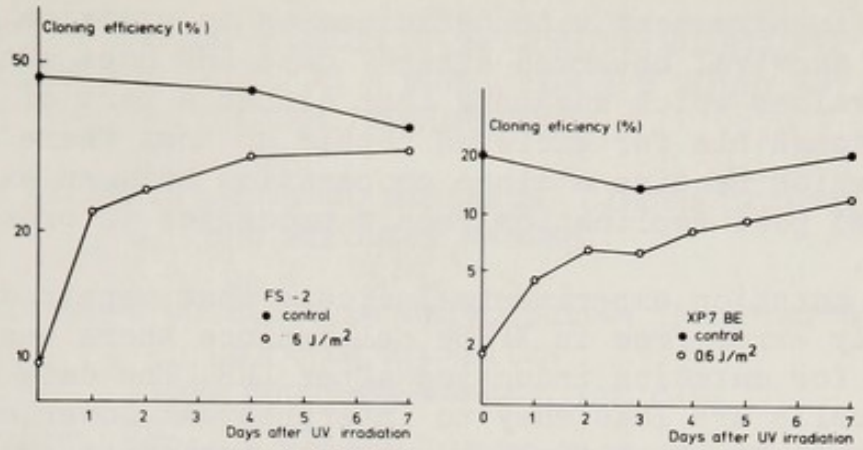

FIGURE 1. Survival of WT and XP7Be cells during 7 days LH.

DMF was 3.7 and 3.9 for two WT strains, while for XP7Be and XP25Ro these figures were, respectively, 2.1 and 1.7. After X-irradiation the DMF was 3.2 for WT and 1.9 for AT5Bi. The effect of LHR on mutation induction by UV was studied in XP7Be and in WT cells (figure 2). For XP7Be no mutation induction could be observed after LHR; for WT a similar or reduced mutant frequency was observed at the lower exposures and a similar or enhanced mutation induction at the higher exposures.

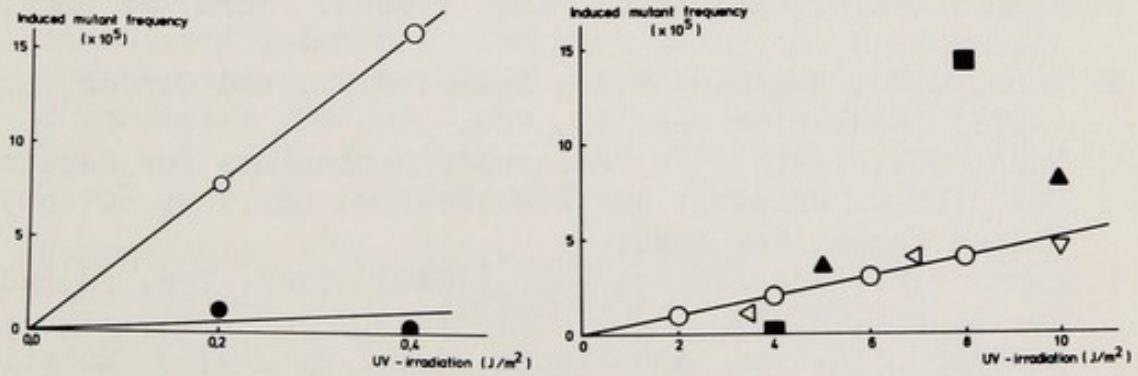

FIGURE 2. Mutation induction in XP7Be (left) and WT (right) before (o) and after (●) 7 days LHR.

DISCUSSION

The data demonstrate that LH-conditions can be maintained for human diploid skin fibroblasts for 7 days and longer. The DMF found after LHR of UV-irradiated and X-irradiated WT cells is substantially higher than the DMF's found for wild type yeast (7), bacteria (2, 9) and density-inhibited Chinese hamster cells (3). The DMF appears to be reduced in XP-cells after UV-irradiation and in AT-cells after X-irradiation,

which is in agreement with deficiencies in excision repair.

The survival observed after 7 days LHR does not reach control values which suggests that either a part of the damage is not accessible for excision repair or that there are lesions which require a close cooperation between excision repair and post replication repair processes in order to be repaired.

The mutation experiments indicate that repair during LH is largely error-free in XP7Be cells since there was no evidence for mutation induction after LHR. The data obtained with WT cells are less easy to interpret. At lower exposures repair could be error-free and at high exposures error-prone. An explanation could be that an error-prone pathway is switched on after massive doses.

ACKNOWLEDGEMENTS

The author is grateful to Miss C. van de Broek for her excellent technical assistance.

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FIXATION AND EXPRESSION OF RECESSIVE MUTATIONS IN MAMMALIAN CELLS AS A MODEL FOR THE STUDY OF CARCINOGENESIS [1]

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ABSTRACT TPA, a powerful tumour promoter is shown to induce sister chromatid exchanges (SCEs), whilst the non-promoting 4-O-Me TPA derivative does not. Two classes of inhibitors of tumour promotion, inhibit such TPA induced SCEs. Thus, TPA is the first agent reported to induce SCEs in the absence of DNA damage, chromosome aberrations and mutagenesis. Since TPA is known to induce several gene functions we would like to speculate that it might also induce enzymes involved in genetic recombination. TPA is also shown to stimulate segregation of a recessive genetic trait, 6-thioguanine resistance, from a heterozygous hybrid cell. Such data support the hypothesis that tumour promotion might be the result of an aberrant mitotic chromosomal segregation event leading to the expression of carcinogen/mutagen induced recessive genetic or epigenetic chromosomal changes.

INTRODUCTION

Experimental carcinogenesis is considered to be a two-step process comprised of "initiation" and "promotion" events.[1,2] Initiators which are radiation or chemical carcinogens, cause rapid irreversible changes probably mutational or epigenetic in origin, which result in a "pre-malignant" state. Promoters are neither significantly carcinogenic[2] or mutagenic[3,4] but greatly increase tumour frequency and shorten the lag-time for tumour appearance when added after an initiator. Thus, tumour promoters complete a process begun by initiators and not vice versa[2]. We propose that initiated cells contain specific, recessive autosomal, somatic mutations and that promotion results in the expression of these mutations by some aberrant chromosomal segregation event which converts the heterozygous (m/+) initiated cell into a homozygous (m/m) or hemizygous (m/o) cell. This would be consistent with the

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2 chercheur qualifié F.N.R.S.

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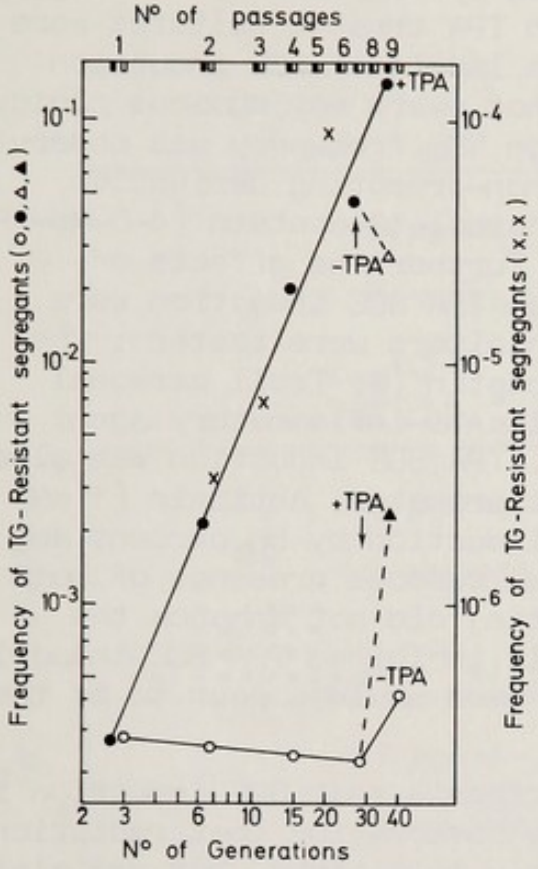
finding that the non-viral malignant (or transformed) phenotype is suppressed on hybridisation with non-transformed cells and that malignant segregants can reappear as a result of chromosome loss[5,6]. Although "recessive mutation" is used this hypothesis is consistent with any model of initiation based on stable recessive genetic or epigenetic changes. Six simple mechanisms exist for the segregation of a recessive mutation : (i) chromosomal rearrangement or deletion and (ii) one step non disjunction, both lead to hemizygosity while (iii) two-step non-disjunction, (iv) random mitotic segregation (v) increase in ploidy plus chromosome loss and (vi) mitotic recombination, all lead to homozygosity. Only the events leading to homozygosity (iii-iv) satisfy the observation that initiation must preceed promotion in order to produce the enhancement of a carcinogenic event. Furthermore, mechanisms (i) and (ii) are discouraged by the fact that chromosome loss or deletion is usually lethal to normal diploid cells[7].

EXPERIMENTAL RESULTS AND DISCUSSION

To test whether or not the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) could promote the segregation of a recessive mutation and a recessive, malignancy-related character, cell hybrids between 6-thioguanine resistant ($\underline{6\text{-TG}}^R$) mouse hepatoma agar growing ($\underline{aga}^+$) cells and primary rat fibroblasts ($\underline{TG}^S$, $\underline{aga}^-$), were used[8]. Both characters are recessive such that the resultant hybrids had a $\underline{TG}^S$,$\underline{aga}^-$ phenotype. Fig. 1 illustrates that the results obtained in the first series of experiments for TPA induced segregation, to $\underline{6\text{-TG}}^R$, are fully consistent with the hypothesis that tumour promoters stimulate chromosomal and/or marker segregations. A similar pattern of TPA stimulated segregation is seen to occur in two different hybrids with widely differing chromosomal stabilities. Furthermore, in the same experiments TPA was shown to similarly stimulate the segregation of $\underline{aga}^+$ cells (results not presented). This experiment shows that TPA is a powerful inducer of segregation of recessive traits from heterozygous cells, although the precise mechanism of this segregation remains to be determined.

In a parallel study mitotic recombination was investigated as a possible mechanism of promotion. It should be emphasised that mitotic recombination in mammalian cells must be extremely rare as it has thus far been impossible to demonstrate in genetic systems[9,10]. Thus, in the absence of a suitable genetic system sister chromatid exchange frequency was considered to be a measure of cellular recombinational activity[11,12]. We have preformed experiments to show that TPA a powerful tumour promoter induces elevated levels of

Figure 1 TPA STIMULATED SEGREGATION TO 6-THIOGUANINE RESISTANCE FROM HETEROZYGOUS CELL HYBRIDS



The isolation and characterisation of cell hybrids derived from 6-thioguanine (TG) resistant mouse hepatoma cells and TG-sensitive rat fibroblasts, has been described previously[8]. Hybrids were seeded at 2.5×10^5 cells per Falcon flask (75 cm^2) in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 50µg/ml gentamycin. TPA was added to give a final concentration of 0.25 µg/ml. This dose was sub-toxic. Each new passage was initiated before confluence was attained : cells were trypsinised, counted (to determine the number of generations) and reseeded again at the initial plating density. At each passage, the control cells (no TPA) and TPA treated cells were withdrawn and plated at densities of 10^5, 10^4, and 10^3 cells per 60mm petri dish and challenged with 2 µg/ml 6-thioguanine. To avoid metabolic cooperation the medium was changed every two days. The colonies were scored after three weeks. The plating efficiency was slightly higher for TPA treated cells (14 versus 11 percent).

Symbols : (1) cell hybrid BS100 : without TPA (o), with TPA (●); arrows indicated 7th passage where (a) TPA was removed from a fraction of TPA-treated culture, which then was grown without TPA for two more passages before the challenge with TG (Δ); (b) TPA was added to a control fraction for two more passages before the addition of TG (▲); (2) Cell hybrid BS181 shows undetectable segregation of TG-resistant cells ($< 10^{-6}$) in the absence of TPA. With TPA however, it segregates with similar kinetics to the BS100 clone (x).

SCEs in V79 chinese hamster lung fibroblasts in the absence of chromosome aberrations. Maximum SCE induction was observed at 1 μg/ml TPA, which was non toxic (our data) and has also been shown to be non-mutagenic for V79 cells[3,4]. The increase in SCE frequency induced by TPA is shown in fig.2. Approximately 18-25% of cells in TPA treated cultures were shown to have exceptionally high levels of SCE induction ("too many to count"). TPA did not exert any obvious ploidy effects. No significant effect on SCE frequency was observed when cells were exposed to the non-promoting derivative 4-O-methyl-12-O-tetradecanoyl-phorbol-13-acetate (4-O-Me-TPA) Fig. 2. To test this hypothesis further the effects of inhibitors of tumour promotion on TPA SCE induction were investigated. Two classes of inhibitors were tested : the antiproteases, antipain and leupeptin (W. Troll personal communication) and the steroidal anti-inflammatory agent fluocinolone acetonide (FA)[13]. TPA SCE induction was clearly inhibited by both classes of antipromoter. Antipain (1 mM) and leupeptin (1 mM) inhibited SCE induction by 56 percent and 76 percent respectively. The simultaneous presence of both inhibitors (antipain and leupeptin) did not enhance the inhibitory effect. FA (1.0 μg/ml) inhibited TPA SCE induction by 100 percent and at the doses used would appear to be the most effective inhibitor.

These data support the hypothesis that SCE induction is related to tumour promotion. The observation that radiation and chemical mutagens are not only initiators, but are also carcinogenic at high doses, is to be expected in this model since relatively high doses of initiators are known to induce SCEs in mammalian cells[14] and mitotic recombination in fungi[15,16] and drosophila[17]. The potential of initiators to produce carcinogenesis at high doses may be masked by their high toxicity. The negligible toxicity of TPA concentrations used to induce SCEs *in vitro* and the low toxicity and the proliferative effect of tumour promoters *in vivo*[2], can be expected to greatly amplify the efficiency of segregation by the mechanism of mitotic recombination. The fact that initiation is a fast irreversible process, whilst promotion is short-lived but cumulative, when the promoter is applied repeatedly[2], is also fully consistent with a model for promotion based on mitotic recombination. Peto *et al* [18] have also discussed the possibility that mitotic recombination resulting in homozygosity might be one of the multiple mechanisms involved in carcinogenesis. Bloom's syndrome, the striking human cancer prone hereditary disease, provides circumstantial evidence favoring our hypothesis, since cells taken from these patients are characterised by very high levels of spontaneous SCEs[19]. Moreover there is compelling cytological evidence of frequent spontaneous mitotic

recombination among homologous chromosomes within these cells (19). Thus, we consider that Bloom's syndrome may be caused by a mutation resulting in a promotion constituitive phenotype.

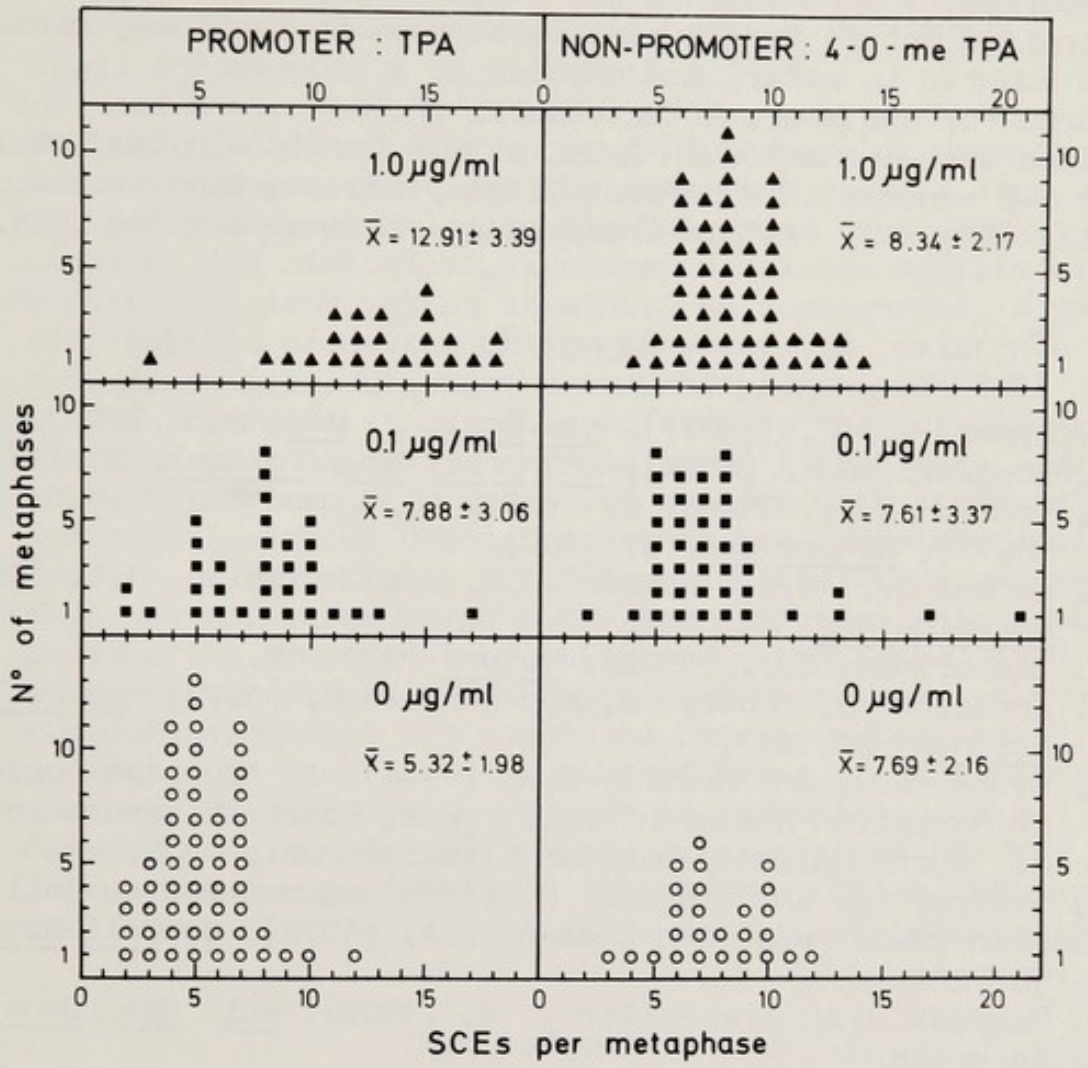


Figure 2 THE DISTRIBUTION OF SCEs IN V79 CELLS TREATED WITH THE TUMOUR PROMOTER TPA AND ITS NON PROMOTING DERIVITIVE 4-O-meTPA.

V79 Chinese-hamster cells were grown in Dulbecco's modification of Eagle's minimal essential medium supplemented with 10 % foetal bovine serum. For SCE analysis exponentially growing cells were exposed simultaneously to bromodeoxyuridine (BUdR) and tumour promoter (10 mg/ml stock solution in acetone) for twenty-eight hours or two generations. Where necessary similar incubations were conducted but with the additional presence of an inhibitor of promotion. Control cultures were treated with the same concentration of acetone as the TPA treated cultures. Until chromosome fixation all cultures were kept in the dark. Approximately twenty-six hours after treatment (2×10^{-7} m) colcemid was added to the cells for two hours

and the mitotic cells isolated by "mitotic shake-off". The cells were treated for eleven minutes with 0.075 M KCl, followed by fixation in methanolactic acid (3:1). Drops (10µl) of the fixed cell suspension were placed on clean microscope slides and air-dried. After twenty-four hours the chromosome preparations were stained with Hoescht 33258 (0.5 µg/ml in deionised water) for 15 minutes at room temperature, rinsed and mounted in water, and exposed to a fluorescent light source for one hour. After removal of the coverslips the slides were incubated in 2xSSC at 60° for 15 minutes, stained for 6-8 minutes in 4 percent Giensa, and examined microscopically (20). All visible metaphases were analysed for SCEs.

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MAMMALIAN CELL MUTAGENESIS AS A BIOLOGICAL CONSEQUENCE OF DNA DAMAGE[1]

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ABSTRACT Evidence that in *E. coli* particular gene products are responsible for the induction of mutations caused by unexcised lesions in DNA comes from elegant biochemical and biological experiments using a battery of repair deficient mutant strains. No such series is yet available in mammalian cells. However, the existence of excision repair deficient xeroderma pigmentosum (XP) strains has made it possible to examine the role of excision repair on mutagenesis in human fibroblasts. Results of our recent comparative studies with these XP and normal strains demonstrate that the excision repair process is essentially error-free and therefore suggest that the mechanism responsible for mutagenesis is some process (or processes) by which cells replicate (or attempt to replicate) DNA containing unexcised lesions. Two general models are suggested for the mutagenesis process. In the first, mismatched bases are introduced into the DNA during the process of replicating on a damaged templa. In the second, saturation of DNA repair processes results in a failure to complete DNA synthesis and this failure leads to deletion-type mutations.

INTRODUCTION

Results of extensive studies over the past decade indicate that the predominant mechanism of ultraviolet radiation (UV)-induced mutagenesis in *Escherichia coli* is a $lexA^+$, $recA^+$-dependent error-prone repair system and recent developments suggest further that this error prone process is not constitutive, but is induced in response to UV radiation or other agents that damage DNA or interrupt its replication ("SOS repair") (1,2). Sedgwick has suggested (3) that the mechanism of induction of mutation by UV in *E. coli* consists

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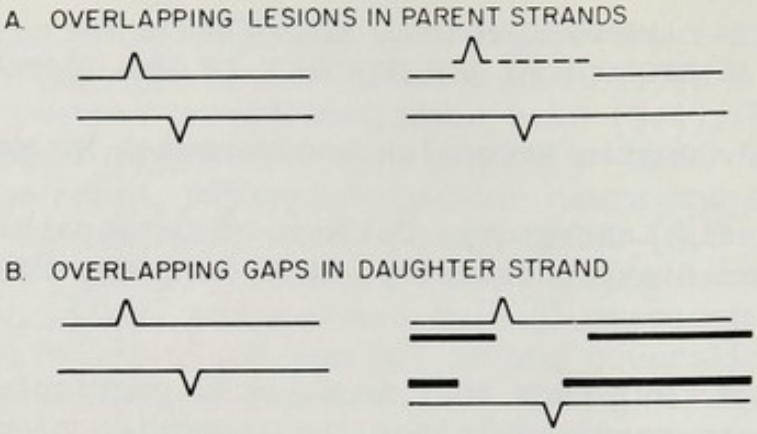

Fig 1. Diagram of two complex repair situations.
A. If excision repair is attempted on one strand, a correct template is lacking on the other.
B. If replication of each parental DNA involves leaving a gap in the daughter strand, overlapping gaps will prevent recombination.

in the misrepair of complex repair situations such as those generated by closely spaced DNA lesions where repair of one lesion involves template DNA containing a second lesion. (See Fig. 1) However, the mechanisms by which mutations are induced in cultured mammalian cells by radiation or chemicals are much less well understood. In this brief overview, we can only highlight some of the current problems and controversies concerning mutagenesis in such cultured cells and the relationship of DNA repair to the mutagenic process.

It should be noted by way of background that the genetic nature of hereditary variation in cultured somatic cells was questioned by Harris in 1971 (4) and by Mezger-Freed in 1972 (5) based on their claims that mutation rates were independent of ploidy, a finding inconsistent with the expected mutational behavior of recessive markers. However, in the intervening years, a number of workers have provided very convincing arguments that true mutational events do take place in cultured somatic cells (6,7) and an impressive amount of evidence now exists in support of this statement (cf. ref. 7). In the light of these data and for the sake of simplicity, we shall, therefore, refer to all isolates of cultured somatic cells which show a stable change in phenotype as "mutants" although we realize that this may not be true in every instance. Similarly, following the lead of Siminovitch (6) and DeMars (7), we will use the term "mutational event" or "induced mutation" to refer to any heritable nucleotide base change, deletion, or rearrangement in the primary structure of DNA whether these heritable changes result from

point or deletion mutations or from chromosomal rearrangements or chromosomal loss.

INDUCIBLE ERROR-PRONE REPAIR IN MAMMALIAN CELLS

Since *E. coli* has often served as an excellent heuristic model for understanding processes in mammalian cells in culture, many laboratories have made intensive efforts during the last few years to determine whether an "SOS-like" inducible, error-prone, complex lesion repair system might also be responsible for mutagenesis in higher cells. To date, we are not aware of any full-length publication that documents the existence of such an "SOS-like" repair process in cultured mammalian cells despite intensive work in many laboratories. D'Ambrosio and Setlow recently reported some biochemical evidence of an inducible post-replication repair process in Chinese hamster cells that is compatible with an "SOS-type" repair system (8) and which is often cited as evidence that such an inducible, error-prone repair process exists. They showed that cells exposed to a small dose of UV or N-acetoxy-2-acetylaminofluorene and several hours later given a large dose of one of these agents were able to carry out "post-replication repair" (i.e., the conversion of low molecular weight newly-synthesized DNA into larger-sized DNA) at a faster rate than cells given only the second (larger) dose. The enhanced rate of this conversion of DNA into higher molecular weight was not observed when cells were treated with cycloheximide between the two exposures, suggesting that it was caused by an inducible repair system which requires protein synthesis. Since "SOS repair" in *E. coli* involves protein synthesis, these results suggest that a similar "SOS-like" repair process operates in a mammalian cell. If so, then such a process is predicted to increase the frequency of mutations induced. Biological experiments to test this prediction have been carried out by Chang, Schultz, and Trosko, of Michigan State University in collaboration with D'Ambrosio and Setlow (9). They found that in Chinese hamster cells subjected to the fractionated doses outlined above, the frequency of induced mutations is not higher than in cells given a single large dose. In fact, it is often lower. Furthermore, cycloheximide treatment instead of preventing an increase in mutation frequency often appeared to cause a two-fold increase, as if inhibiting an error-free process, not the error-prone process predicted by the "SOS" hypothesis. It should be noted that all of these experiments are further complicated by the fact the cycloheximide has been shown to inhibit both DNA and protein synthesis in

eucaryotes (10,11) and therefore, one cannot conclude that its observed inhibition of the elongation of newly-synthesized DNA necessarily results from its inhibition of protein synthesis alone. Setlow and Grist reported (12) a similar enhancement in variant XP cells of the rate of converting low molecular weight DNA into larger molecules after UV irradiation (13). We have investigated the effect of their fractionated dose protocol in XP variant cells and did not observe any increase in survival compared to cells given the single larger dose (unpublished observations).

EVIDENCE THAT EXCISION IS ERROR FREE

By what process do mutations arise in cultured mammalian cells following treatment with mutagenic agents? Evidence from our experiments comparing the frequency of mutations induced by UV in diploid human skin fibroblasts derived from XP patients and normal persons indicate that they are not caused by excision repair. XP-derived fibroblasts have low rates of excision repair of UV-induced pyrimidine dimers in their DNA as Cleaver showed some nine years ago (14). The rate of excision repair of the various excision deficient XP

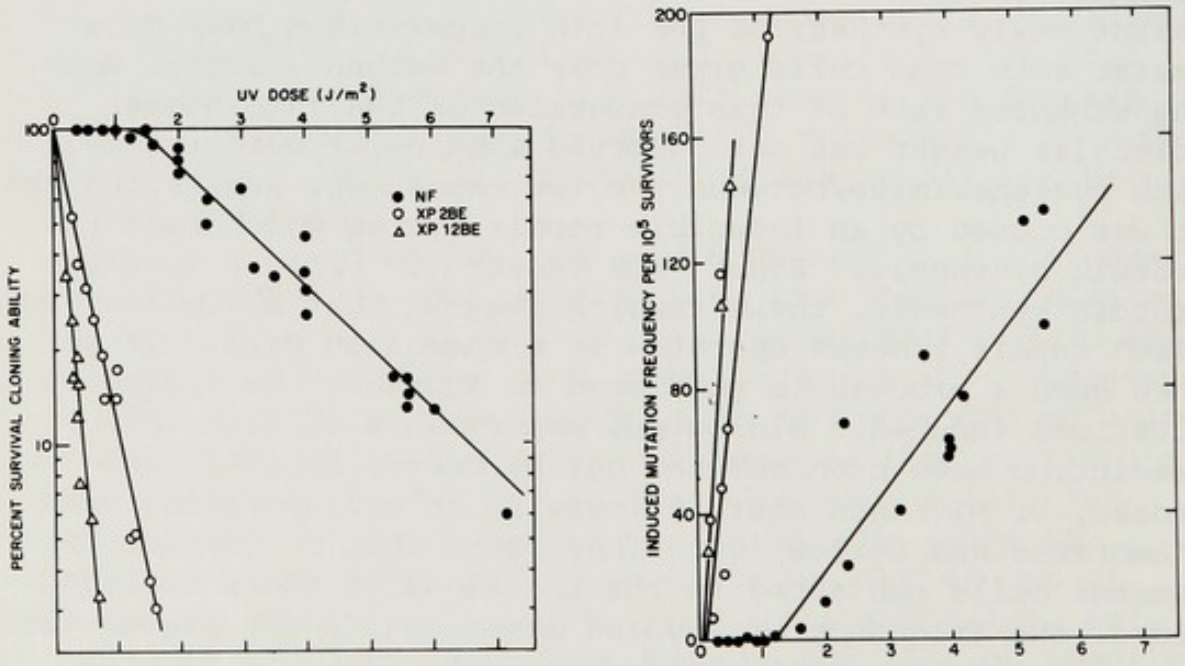

Fig 2. **Right:** Percent survival, as a function of UV dose, of the colony-forming ability of various strains of human skin fibroblasts with different capacities for DNA repair. **Left:** Frequency of mutations to azaguanine resistance, as a function of UV dose, in these same fibroblasts.

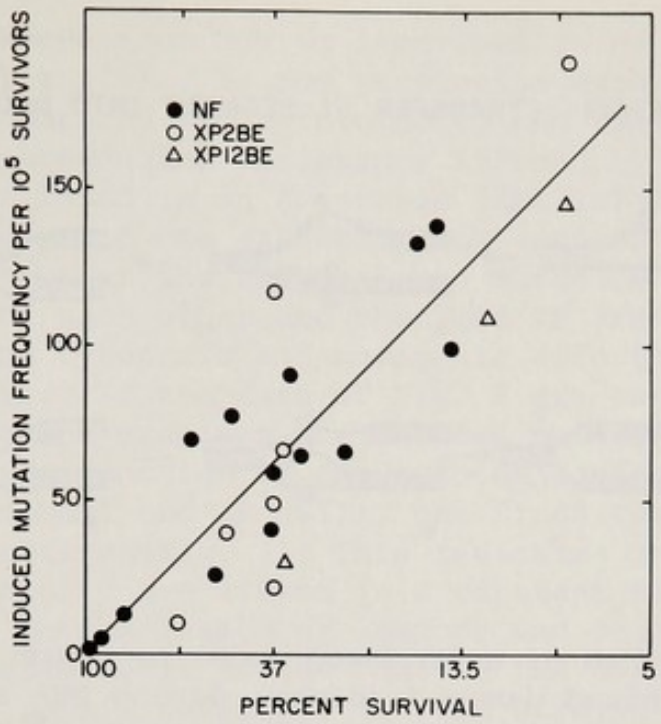

Fig 3. Data from Fig 2 on the frequency of induced mutations per 10^5 survivors replotted as a function of the percent survival. The fact that all the points lie along a single line suggests that the mechanism of mutagenesis is similar in these XP cells and normal cells.

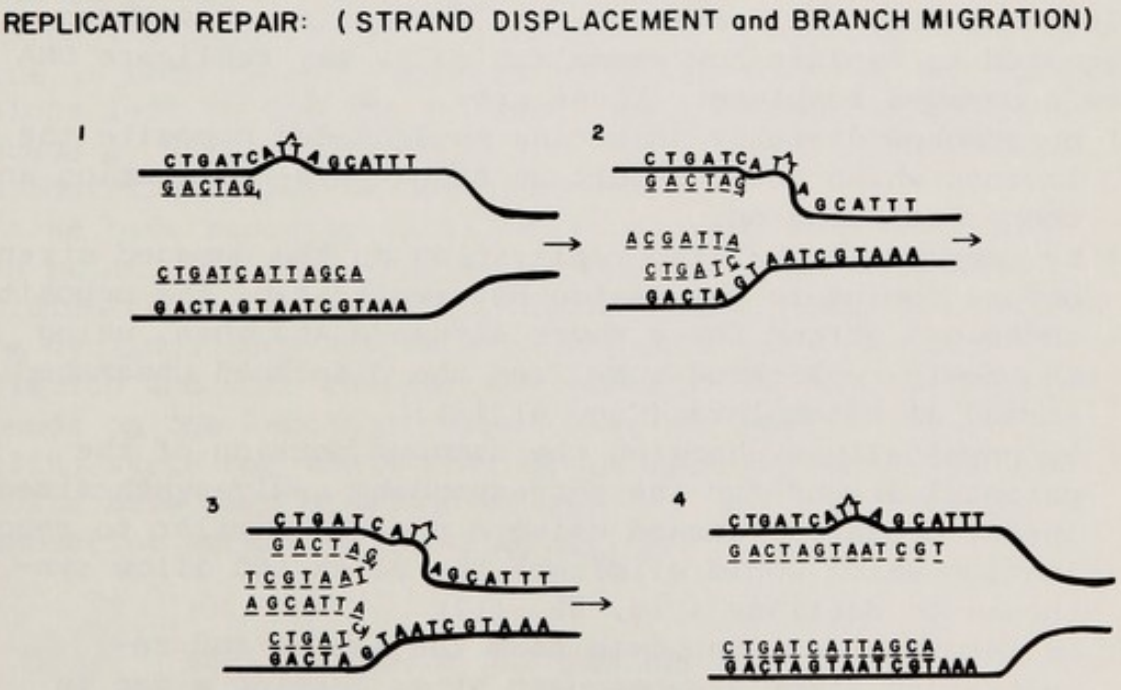

Fig 4. Diagram of how a cell which is replicating DNA containing unexcised damage might "by-pass" the damage without having to make use of the damaged section as a template. From Higgins *et al.* (16).

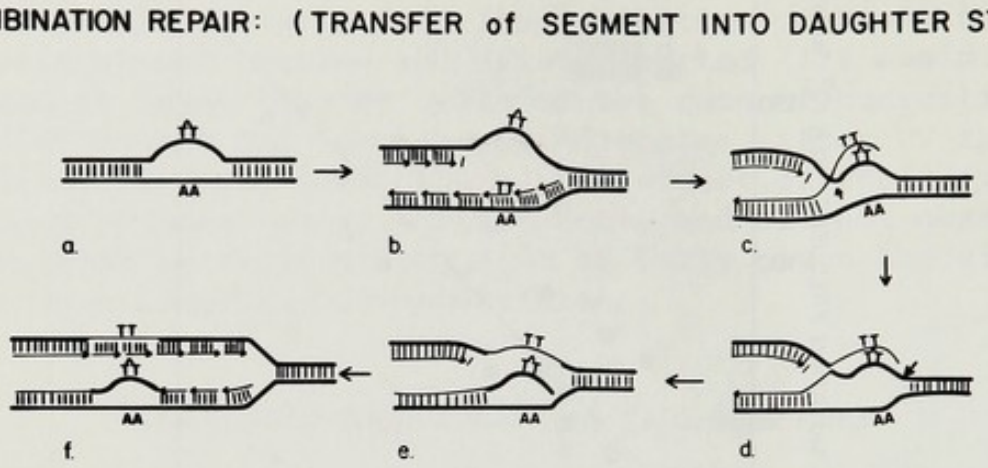

Fig 5. Diagram of a proposed model by which a cell could "by-pass" unexcised damaged (dimer) during DNA synthesis by transferring the lesion into a daughter strand (17). See refs. 19 and 20 for data indicating that such transfer of dimers can occur.

introduced. The *second* considers that the mutations derive from the failure to complete DNA synthesis as a result of saturation of repair mechanisms and, therefore, represent deletions. For the former, several mechanisms have been suggested to explain how mammalian cells may replicate DNA from a damaged template. These are:

(1) by somehow directly inserting nucleotides opposite the lesions which could result in direct mis-replication and then continuing on;
(2) by temporarily halting replication on the damaged strand before coming to the lesion but replicating the opposite undamaged strand for a short distance and then, using the newly-replicated copy from the displaced undamaged strand as a template (Fig. 4)(16);
(3) by physically exchanging the *damaged* portion of the parental strand for the corresponding newly-synthesized intact daughter strand using a process similar to recombination which would eliminate the block and allow synthesis to continue (Fig. 5) (17)
(4) by interrupting synthesis near the lesion and re-initiating it at a subsequent site, leaving a gap in the vicinity of the damaged site which could subsequently be filled either by two *de novo* synthesis or by recombination between daughter and parental strands.

cell strains ranges from 50% to less than 2% of that found with normal cells (15). In the particular experiments shown in Fig. 2, we compared the cytotoxicity and the frequency of mutations to 8-azaguanine resistance induced by UV in normal cells with that found in an XP strain (XP12BE) with no excision capacity and one (XP2BE) which excises at a rate 16% of normal. Note that the survival and mutation curves are mirror images of each other and that the XP are far more sensitive to the cytotoxic and mutagenic effects of UV than are normal cells. If the data of Fig. 2 are taken and replotted so that one examines the induced mutation frequency per survivor as a function of the cytotoxic effect of exposure to UV in normal and XP cells, one finds that the data falls along one line (Fig. 3). This indicates that cytotoxicity and mutagenicity are linked in a constant ratio and could be caused by a common lesion or lesions and suggests that the mechanism of mutagenesis is the same in the normal and in these excision repair deficient XP cell strains. It is apparent that the difference between these cell strains is the dose of radiation required to obtain a particular percent survival (and corresponding induction of mutations) and that this is much lower in XP cells than in normal cells. The fact that the population of normal cells with the high rate of excision repair is the one with the lower mutation frequency per dose argues that the excision repair process is essentially error-free. Now, if excision repair is not causing the majority of the mutations, it must be that mutations in both XP and normal cells arise from the non-excised lesions left in the DNA -- most likely as a result of some process by which cells replicate (or attempt to replicate) DNA containing such UV-induced lesions.

We have recently obtained additional data which bear on this point. We find that if XP cells with some residual excision repair capacity and normal human cells are blocked from cycling, they are able to tolerate large doses of UV radiation and that the percent survival and the mutagenicity depends on the length of time cells are held in the non-cycling state and their rate of excision repair. Similar results have been obtained by Simons and his coworkers. (manuscript submitted for publication).

REPLICATION OF DNA CONTAINING LESIONS

Two general models can be proposed to account for DNA changes in mammalian cells that result in the mutagenic effects of UV and certain classes of chemicals. The *first* postulates that in the process of replicating DNA containing UV photoproducts or chemical damage, mismatched bases are

The first could introduce errors, e.g., base pair changes or frame shifts, since a non-instructive template is used for synthesis. The second ought to be "error free" since an undamaged template is substituted for the damaged one as is obvious from the model. However, nothing is yet known about the *fidelity* of the polymerases(s) involved in carrying out the proposed replication. Since the third model involves endonucleases and ligases and does not depend upon accurate base pairing by a polymerase, it could result in the loss of small amounts of DNA (deletion mutations) or even larger amounts (chromosome aberrations). Neither the second nor the third model would operate efficiently in situations similar to those diagrammed in Fig. 1. The fourth model, i.e., leaving gaps which must be filled *de novo* or by recombination holds potential for the production of mutations either by substituting incorrect bases across from the non-instructive bases or by the loss of genetic information during recombination.

While there is some physical evidence for all four processes in mammalian cells there is no definitive evidence to indicate whether one of them or some other process results in mutations. Furthermore, it is possible, indeed likely, that several such mechanisms ordinarily co-exist in cells to allow replication of DNA containing lesions since several processes of these types occur in *E. coli*.

The second model is proposed by Roberts (20) and is based on his observations that in Chinese hamster cells, cell death, induction of mutations, and chromosome damage are all closely associated and caffeine causes an increase in all three parameters. Therefore, either all three are a manifestation of one caffeine sensitive process or they result from three processes, all of which show a similar caffeine sensitivity. A diagramatic model of Roberts' results is shown in Fig. 6. In this experiment the rate of DNA synthesis is depressed in a dose-dependent manner by the chemical agent used but this is compensated for by a longer period of DNA synthesis and a delay in mitosis which is proportional to the dose of the agent. In Fig. 7, we see diagramatically the result of the addition of a non-toxic dose of caffeine to this same experiment. The rate of synthesis is no longer depressed, no delay in mitosis is observed, and the length of the DNA synthesized now closely approximates the distance between lesions in the DNA. Thus, caffeine allows the synthesis of low molecular weight DNA between all the lesions but seems to block a DNA repair process which would have allowed the lesions to be circumvented slowly and eventually resulted in the synthesis of DNA of high molecular weight. Evidence that this is in fact the correct interpretation is shown by the fact that in the presence of caffeine the rate

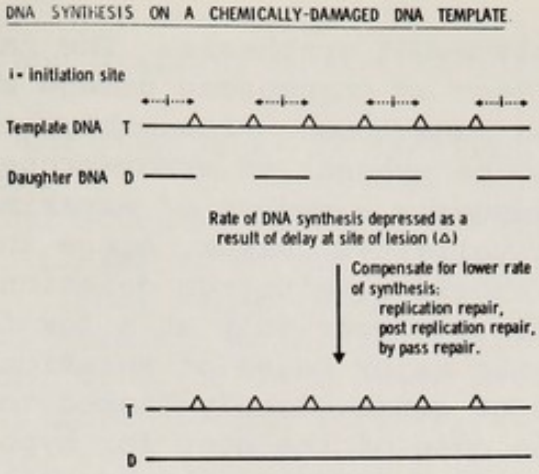

Fig 6. Diagramatic representation of Roberts' experiments measuring DNA synthesis in Chinese hamster cells treated with N-methyl-N-nitrosourea, cis platinum (II) dichloride or 7-bromomethylbenz(a)anthracene. (From ref. 20).

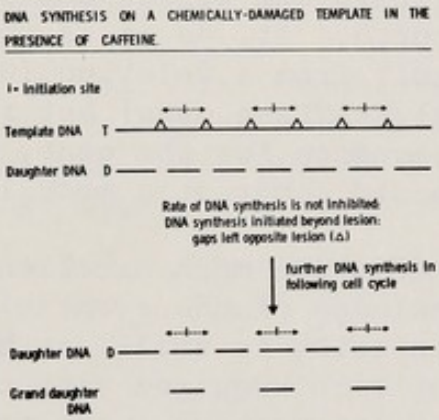

Fig 7. Diagramatic representation of Roberts' experiments measuring DNA synthesis in Chinese hamster cells treated as in Fig 6 and with non-toxic doses of caffeine added. (From ref. 20).

of DNA synthesis is greatly reduced in the next cell cycle (synthesis of granddaughter DNA) presumably as a consequence of the amplification of lesions in DNA in the synthesis of daughter DNA. Futhermore, cells treated with mutagen plus caffeine (as in Fig. 7) show extremely high frequencies of chromosome aberrations (e.g., 66%) and cells with treated with mutagen alone (as in Fig. 6) show lower (e.g., 48%) frequencies of chromosome aberrations. Such results are an expected consequence of the failure to complete DNA synthesis. Other studies by Roberts with HeLa cells gave similar results, except that these cells had no caffeine-sensitive repair process. Thus, he suggests that all three phenomenon, viz., cell death, induction of mutations and chromosome damage result from the saturation of the DNA repair systems which

results in incomplete DNA synthesis. The failure to complete DNA synthesis is seen as chromosome damage which is detected mainly as deletion mutations. Presumably, other types of mutations may also be present if error-prone repair exists.

This model suggests a number of experiments which may serve to test its validity. First, since the majority of mutations formed should result from deletions, back mutation of mutant cells should occur only at a low frequency. Second, if deletions are the major cause of mutations, one should also see the loss of genes closely linked to the gene one is measuring. In the case of the gene for hypoxanthine-guanine-phosoribosyltransferase (HPRT) in human cells, glucose-6-phosphate dehydrogenase is such a gene and we are currently testing our HPRT mutants for its presence. Third, extensive chromosome damage should be exhibited by cells treated with doses of mutagen that are only moderately mutagenic. Roberts has seen this in V79 cells but we are unaware of such studies by other workers. Fourth, if, as it is postulated, ouabain resistance cannot result from a deletion (which would leave the cell without a Na+/K+ATPase gene) but rather must result from a changed base sequence for the gene, mutations to ouabain resistance should occur at a much lower frequency than HPRT mutations.

It is obvious that while much excellent work has been done, our present knowledge of the event(s) responsible for mutations in mammalian cells is still, at best, theoretical. Further experiments will be required with cells for various mutagens, with a number of markers, with mutants defective in various repair processes as well as with repair inhibitors before we can determine whether one or more theories or some other is correct.

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RELATION BETWEEN DNA REPAIR, CHROMOSOME ABERRATIONS, AND SISTER CHROMATID EXCHANGES

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ABSTRACT Ordinary chromosome aberrations and sister chromatid exchanges (SCEs), which are two types of cytogenetic endpoints that are currently being used to determine the effects of mutagens and carcinogens on chromosomes, differ in several aspects. A) Double stranded DNA breaks, which are induced by X Rays, lead to aberrations but not to SCEs. B) Ordinary chromosome aberrations that are induced by ultraviolet light are photoreactivable, whereas we now have found in chick embryo cells that even though thymine dimers are removed by PR light, the yield of UV-induced SCEs is unaffected. C) Aberrations induced by UV or "UV-like," but not "X-ray-like" mutagens are higher in XP cells than in normal cells, whereas SCEs are higher in XP cells after treatment with all three types of mutagens. D) The aberration yields are related to the amount of excision repair as measured by unscheduled DNA synthesis or repair replication whereas SCE yields are not. E) Aberrations are increased by application of caffeine after treatment with UV light or chemical mutagens but SCEs are not, and in post-replication defective XP variant cells, UV-induced SCE levels are as in normal cells. Taken as a whole these data indicate that although aberrations are dependent upon excision repair and post-replication repair as measured in ordinary experiments, SCEs are not. The lesions that result in SCEs do not seem to be thymine dimers or alkylation products at the N^7 position of guanine. Since SCEs are induced by long-lived lesions that disappear with time, minor photoproducts, or alkylation products, that are not measured in excision repair or post-replication repair experiments seem to be the lesions responsible for SCE formation, which is contrary to the findings for chromosome aberrations.

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INTRODUCTION

When cells are treated with mutagenic carcinogens the genetic material in the nucleus of the cell provides the most important target that is subjected to damage. If this damage is in somatic cells it can affect the exposed individual himself by inducing cancer, whereas if it is in the germ cells it can either induce sterility in the individual or induce genetic defects that could possibly affect future generations.

The mutagens can cause effects observed either at individual gene loci or in the whole genome. Included in the latter are gross chromosomal aberrations, which because of the difference in size between a gene and a genome, are about 10^5 times more frequent than are effects noted at individual loci. For this reason, more than any other, the study of chromosome aberrations has been a favorite way to study the mechanism(s) of action of mutagens in higher organisms.

Recently, however, a new method has become available which is far more sensitive than even aberrations for the detection of cytogenetic effects of mutagens. This is the formation of sister chromatid exchanges (SCEs) that can be observed in chromosomes having sister chromatids that are chemically different from one another (1,2,3,4,5).

CHROMOSOME ABERRATIONS

Treatment with Ionizing Radiations. In the past the mutagen most studied for the induction of chromosome aberrations has been ionizing radiation, which can induce aberrations throughout the cell cycle (6,7). The types of aberrations and the numbers induced, however, vary from cell stage to cell stage. Thus, if cells are irradiated in G_1 before the chromosomes replicate, then the chromosomes, which consist of a DNA double helix and its associated protein, react as though they were single structures and give complete breaks. These breaks can then undergo repair either by rejoining with another nearby broken end to form an aberration (misrepair), or by restituting, which is rejoining in the original configuration and so not giving rise to an aberration. The breaks can also remain open or unrepaired. Later when the cell enters S and the chromosomes replicate, they will contain two daughter chromatids; both of which are similarly affected. Since the whole chromosome is involved these are termed chromosome aberrations to distinguish them from the types of aberrations found when cells are irradiated in S

or G_2. In these cases, the chromosomes react to radiation as though already doubled and the chromatid is the unit of breakage leading to chromatid aberrations (8). The ends can undergo the same types of repair (rejoining) as observed for chromosome aberrations.

Treatment With Chemical Mutagens and UV. Chemical mutagens and the non-ionizing radiation, ultraviolet light (UV), however, differ from ionizing radiation in that they are generally unable to induce full chromosome aberrations, irrespective of the cell stage irradiated. If a cell is treated in G_1 with these mutagens, then a long-lived lesion is formed that can lead to the production of a chromatid aberration when the cell passes through S (9). Treatment within S itself can also lead to the induction of chromatid aberrations, but only in those regions of the chromosomes that have yet to replicate, for in replicated regions of S cells, or in already replicated G_2 cells, these agents do not induce aberrations that can be observed at the first succeeding metaphase. Those lesions that are induced in replicated regions of chromosomes, however, if unremoved by repair processes, can give rise to chromatid aberrations in succeeding cell cycles when the cell passes into S and the chromosomes replicate (9). Similarly, any lesion induced in G_1 that was both unrepaired and did not give rise to a chromatid break in the first S could survive and lead to aberration formation at subsequent rounds of replication. Thus, these aberrations, unlike those produced by ionizing radiations, are produced by errors of replication, and the agents that induce them are considered S-dependent (10).

SISTER CHROMATID EXCHANGES

Even though ordinary chromosome aberrations occur much more frequently than do point mutations, they have not been the ideal cytogenetic object for study. Part of the reason for this is that they can be quite complex and difficult to score. Furthermore, large numbers of cells must be tediously analyzed in order to gain large enough numbers for statistical analysis. The new techniques for making chromosomes containing sister chromatids that are chemically different from one another, and so stain differentially, has resulted in our being able to analyze sister chromatid exchantes with far greater ease and precision than was possible in autoradiographic studies.

SCEs VS ABERRATIONS

Although sister chromatid exchanges affect both chromatids similarly and thus appear to be analogous to ordinary chromosome aberrations, there are a great many differences between the two, and studies that have been carried out on the relation between repair and the induction of these two cytogenetic endpoints have indicated that the lesions and/or the cellular processes that give rise to aberrations and SCEs are different (11).

X-Ray Induced Lesions. SCEs, unlike ordinary chromosome aberrations, are relatively insensitive to induction by ionizing radiation (12,13) and are not induced throughout the whole cell cycle. Thus if cells are irradiated in G_1 or G_2 either no increase in SCEs is observed or only a very slight one after extremely high doses that produce a plethora of chromosome aberrations (13). Somewhat more SCEs can be induced if cells are irradiated in S but even then far more aberrations are induced.

The most important lesion for the induction of aberrations with radiation seems to be the double stranded break in DNA (10). The argument for this comes from experiments in which it was found that high LET radiations are more efficient at inducing aberrations than are low LET radiations. The high LET radiations, which produce higher ion densities along their tracks, induce a higher ratio of double stranded breaks to other lesions such as single stranded breaks and base damage. Therefore the sensitivity seems to increase as the number of double stranded breaks increase. Further, treatment with chemicals and with ultraviolet light that produce mainly base damage (and perhaps secondarily even single strand breaks) lead to aberrations only in S (14), whereas ionizing radiations, which give rise to double strand breaks, can induce aberrations both in G_1 and G_2 as well as S (6,7). Another inference for the involvement of double stranded breaks comes from experiments in Vicia faba where no excision repair is found after X rays. Nevertheless, in Vicia X rays induce aberrations in G_1 and G_2, indicating that excision repair, and thus base damage, is not involved in the formation of the aberrations (15). Consistent with this notion is the observation that although chemicals and UV can induce an excision repair response as measured by unscheduled synthesis in G_1 and G_2 cells, these mutagens, which do not produce double strand breaks, do not induce aberrations that are formed in these parts of the cell cycle. It therefore appears

that double strand breaks cause the G_1 and G_2 aberrations. It should be noted that, as now would be expected, after ionizing radiation, no long-lived lesions are formed which give rise to chromatid aberrations at the next division.

The SCEs on the contrary are only formed when the cells enter S (12,27) and are produced more proficiently by mutagens that induce base damage (11,13,16,17). The few SCEs that can be observed following irradiation of S cells (13) are very likely those induced by the small proportion of base damage induced by X rays themselves.

SCEs and aberrations seem to vary in many other ways, which can be determined from studies of the differences in their production by a variety of mutagens that induce different lesions, or by their production in cells under conditions where various repair processes are absent or modified.

UV-Induced Lesions. If one studies UV-induced chromosome aberrations, it is found that they, like chemically induced aberrations (18) are caused by long-lived lesions that are converted to aberrations when the cell passes through S. Since pyrimidine dimers are the most common UV-induced lesion, this is the most likely candidate to be responsible for aberration formation. Indeed, experiments with Xenopus cells (14), which contain the photoreactivating (PR) enzyme that can monomerize dimers, have shown that PR light can remove the lesions responsible for the aberrations. This constitutes strong evidence that thymine dimers are responsible for most of the aberrations induced by UV light.

With SCEs the story seems to be somewhat different since UV experiments carried out with Chinese hamster cells show that the yield of SCEs is the same in cells that have different excision repair capacities (12). The same conclusion can be reached from experiments with xeroderma pigmentosum cells that vary in their capacity to carry out unscheduled DNA synthesis (19). Cells from all five complementation groups and from an XP variant differ in their ability to carry out excision repair. The range is from less than 5% repair capacity to normal. There is no consistent relation between the amount of repair and the sensitivity manifested as an increased yield of SCEs. The conclusion from these results seemed anomalous since it had been reported that in Potorous cells, which have the PR enzyme, photoreactivating light can decrease the yield of SCEs, indicating that thymine dimers, which are excisable lesions, could indeed cause SCEs (20). We, however, have been unable to repeat these experiments with Potorous cells, which require up to 6 hr exposure in PR light to see any loss of dimers. Furthermore, we have carried out

photoreactivation experiments on chick embryo cells in which it is possible to measure not only the SCEs but also the loss of dimers themselves. The experiment showed that in these cells photoreactivating light does not reduce the yield of UV-induced SCEs even though it does reduce the residual amount of dimers in the DNA.

This result in conjunction with those obtained in the early experiments with hamster (12) and human (19) cells that have differing repair capacities lead us to believe that although thymine dimers are the most common UV-induced lesion, they are not the lesion that causes SCE formation. It seems that one (or more) of the less common photolesions is responsible for the SCEs.

Chemically-Induced Lesions. The effects noted above are similar to the results obtained with alkylating agents on SCEs induced in normal human and xeroderma pigmentosum cells (11). There, too, it was found that the induction of SCEs was independent of the cell's ability to carry out measurable excision repair, indicating that a minor component of the damage was responsible for SCEs. This is contrasted to the induction of chromosome aberrations, which is directly related to the cells ability to carry out repair. That is, after X-ray treatment or treatment with a variety of alkylating agents, xeroderma pigmentosum cells are able to carry out a normal level of excision repair measured by unscheduled DNA synthesis or repair replication (21,22). The numbers of aberrations found in these cells is the same as in normal cells. After UV exposure (23) or treatment with 4NQO (22), however, excision repair was reduced in XP cells and the aberrations high. It thus appears that the lesions that are responsible for measurable repair after treatment with chemicals are also responsible for aberrations. With SCEs, however, the story is different. The yield of SCEs is high in XP cells whether or not they carry out a low or a normal level of excision repair (11). Since the lesions induced by chemical agents that can lead to SCEs are long-lived lesions (18,27), these XP results would indicate that unexcised lesions lead to SCEs and that these lesions are a small fraction of the total lesions so that they do not affect the relatively crude measurement of unscheduled DNA synthesis. That is, after a seemingly normal amount of excision repair has been carried out, the lesion(s) responsible for SCE induction remains behind.

DISCUSSION

On several occasions it has been postulated that SCE induction itself is dependent upon DNA repair processes (12,16, 24,25,26), and, in particular, because excision repair did not seem to be involved, on post-replication repair (12). Experiments to test this were carried out in plants with thiotepa (27) and in Chinese hamster cells with thiothepa, EMS, MMS, DMS, 4NQO, and MMC (28). Treatments with high concentrations of these chemicals can induce chromosome aberrations. If the chemical treatment is followed by the addition of caffeine, which inhibits post-replication repair, the yield is increased (29). This implicates post-replication repair processes in the formation of chromosome aberrations by these compounds. At low concentrations the mutagens can induce large numbers of SCEs, but the yield of SCEs is not increased by post-treatments with caffeine, indicating that caffeine sensitive repair processes are not involved in their formation. A finding consistent with this is that post-replication defective cells from a xeroderma variant had the same yield of UV-induced SCEs as did normal cells (19).

In all of these cases, aberrations and SCEs responded differently to the treatments, indicating that there are different lesions and/or mechanisms by which the lesions lead to the two cytogenetic effects. This conclusion is supported by the results of several additional types of observations. For instance, SCEs are produced in profusion by concentrations of chemical mutagens that are well below the apparent threshold for the induction of aberrations (13,16,17,30), and studies with cells from patients with three different chromosome breakage syndromes, Bloom's syndrome, Fanconi's anemia, and ataxia telangiectasia, show that SCEs are not consistently correlated with aberrations. Bloom's cells have an extremely high number of SCEs (31), whereas the other two are normal in this respect (31,32,33). Further, Fanconi's anemia cells, which have been shown to be defective in crosslink repair (34, 35) and have more aberrations after treatment with mitomycin C (34) than do normal cells, have a reduced MMC response as far as SCEs are concerned, i.e. fewer SCEs are induced by MMC in these cells than in normal cells (33).

This latter result not only adds evidence that aberrations and SCEs result from different lesions, but indicate that interstrand DNA crosslinks are unlikely to be responsible for SCEs. In general, the experiments on the differences between aberrations and SCEs have given a fair indication of what types of lesions give rise to aberrations, but they have

only been able to indicate which lesions are not involved in SCE formation. These may be noted as follows: the difference in response of aberrations and SCEs to X rays (and incorporated radioisotopes) indicates that double stranded breaks are not a prime lesion that can lead to SCE formation. The difference in response to UV radiation in cells of different excision repair and photoreactivation capacities indicates that thymine dimers do not lead to SCEs. The difference in response of XP cells to chemicals that can elicit measurable excision repair indicates that alkylation of the major site of attack in DNA, the N^7 position of guanine, does not lead to SCEs.

Recent experiments on the repair in XP cells of alkylation at the O^6 position of guanine have raised the possibility that there might be a causal relation between SCE induction and unexcised O^6 alkyl guanine (36). A major alkylation product after treatment of cells with many alkylation agents is N^7 alkyl guanine. Thus, after treatment with methyl nitrosourea both normal and XP cells have 10 times as much N^7 methyl guanine in their DNA than O^6 methyl guanine. The N^7 methyl guanine is readily excised not only from the normal cells but also from the XP cells, as might be expected since methyl nitrosourea elicits a normal excision repair response in XP cells. If XP and normal cells are exposed to ethyl nitrosourea, however, a difference is found in their ability to excise a major fraction of the damage. With ethylnitrosourea 40% of the alkylations in guanine are at the O^6 position and 60% at the N^7 position. The O^6 alkylguanine is removed from DNA of normal cells as fast or even faster than is N^7 alkylguanine, but in XP cells the N^7 alkylguanine is removed much faster than O^6 alkylguanine. This defect in the ability of XP cells to remove a lesion that is usually a minor alkylation product in DNA raises the possibility that attacks at the O^6 position of guanine might be lesions that can lead to SCE formation. Consistent with this notion is the observation that mitomycin C is a potent inducer of SCEs (13,16,37). Since the Fanconi's anemia experiments mentioned above indicate that crosslinks are not likely to be responsible, other MMC lesions become reasonable candidates and interestingly enough, the main monoadduct of MMC to guanine occurs at the O^6 position (38).

A further inference that damage at the O^6 position of guanine could give rise to SCEs is made from a consideration of the relation of SCEs to mutation. It has previously been noted that SCEs, unlike aberrations, are representative of events, like mutations that are compatible with cell survival (11). A positive correlation has now been found between chemically-induced SCEs and mutation (39), a process which had earlier been found to be caused mainly by alkylation at

the O^6 position of guanine. The possibility of alkylation at other positions in DNA, however, cannot be overlooked (40).

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DNA REPAIR IN DIFFERENTIATION[1]

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ABSTRACT Some data have been interpreted as providing evidence for altered DNA repair capacities concomitant with cell differentiation. This proposal is supported by certain experimental models. In addition, information available from DNA repair mutants, from some lower eukaryotes and from systems such as immunoglobulin gene movement suggest that repair systems may play essential roles in effecting or stabilizing patterns of gene expression required for differentiation.

INTRODUCTION

There are two senses in which DNA repair systems could be related to cell differentiation: alterations in repair capacities concomitant with changing developmental state and direct dependence of differentiation on repair as such to effect or stabilize particular patterns of gene expression.

Programmed variation of gene expression is central to the multivalent process of differentiation. In certain classes of cells in metazoa the program results in essentially terminal suppression of DNA replication. By virtue of the partial overlap which exists between repair and replication enzyme sets, it could be anticipated that some alteration in repair functions might accompany terminal differentiation. Such an event could be either a trivial, passive phenomenon, or it could be important to the functional requirements of the cell in this state. In either case this view looks upon changes in repair capacity as concomitants or consequences of the differentiated mode.

Another possibility is that repair functions may be directly responsible for certain facets of the process itself. DNA repair is normally monitored by observing the response to extrinsic damaging agents. Active participation by repair systems would imply that they perform essential functions independent of the presence of extrinsically generated DNA damage. Apart from involvement in replication itself, this consideration suggests the possibility that repair systems

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could function in genome modification, either to produce particular patterns of gene expression or to stabilize same. Seen against the dogma of a totipotential genome and nuclear transplantation experiments generating whole organisms, this concept is a challenging one. Recent data suggest it is indeed important, at least in special cases.

UNSCHEDULED DNA SYNTHESIS AND DNA STRAND BREAKS

A number of studies has been concerned with apparent alterations in DNA repair capacity as measured by radiation- or chemically-induced unscheduled DNA synthesis. Thus Hahn *et al* (1) found that the rate of repair synthesis in fully developed myotubes 8-10 days in culture following explantation from rat embryos was much lower than in young myoblasts or in fibroblasts explanted at the same time. Primary heart muscle cells from adult rats are reported to exhibit no repair synthesis, in contrast to cells from newborn animals (2). The absence of repair-synthesis in UV-irradiated, dormant chicken erythrocytes has been reported (3); repair was reactivated following fusion with HeLa cells or fibroblasts. The accumulation of DNA strand breaks (4) and very low levels of repair synthesis (5) have been reported for rodent cerebellar neurons, although one study of dorsal root ganglion repair gave positive results (6). Spermatozoa appear to have no capacity for rejoining breaks, whereas breaks are repaired efficiently in spermatogonia (7). Early mammalian embryos possess enzymes required for excision repair (8). The response to chemical damage in cycling and non-cycling tumor cells, however, is complex (9), depending on cell type and culture conditions.

During lens development in the chick embryo, the epithelial cells which underline the posterior capsule differentiate in the annular pad into fibers. This is a terminal process, since ultimately the fiber nucleus will degenerate. Counis *et al* (10) have recently shown that at least one DNA repair process is impaired with terminal differentiation.

DIFFERENTIATION IN THE NERVOUS SYSTEM

Despite the suggestion of decreased repair capacity in a variety of differentiated cells, it has long been known that embryonic tissues, and proliferative tissues in the mammalian adult (e.g. lymphoid tissue, gut epithelium) are more sensitive to ionizing radiation than differentiated tissues (11). Thus, while replicating neurons in the neonatal rodent cerebellar cortex are sensitive (12), adult neurons of the central and peripheral nervous systems are

relatively resistant to ionizing radiation (13).

Recently, studies of UV and ionizing radiation effects on differentiating, organotypic cultures of embryonic mouse brain have demonstrated the resistance of neurons to both agents, when compared to glia (14). It is the non-replicating, differentiated neurons which survive best, replicating neurons surviving less well than some replicating glia. The proportion of replicating glia surviving moderate UV doses was higher than that surviving ionizing radiation. Unscheduled DNA synthesis following UV radiation occurred in both neurons and glia. However, in two respects the response was different: (1) neurons had a lower grain density per nucleus than glia; (2) while at appropriate UV doses all glia exhibited either replication or repair, a subpopulation of neurons showed no labelling at all. Subsequent examination of the relationship between grain density and nuclear area suggests this pattern may in part reflect the condensed nuclei in neurons and the spread nuclei in glia (Dambergs and Kidson unpublished).

These studies confirm in cell culture the differential sensitivity to ionizing radiation shown by proliferating and differentiated brain cells *in vivo*, and demonstrate the differential behaviour of neurons and glia with respect to DNA replication and repair following UV irradiation. The most important factor determining response to radiation appears to be the status of DNA replication in the cell, cells which replicate being more sensitive, whatever their apparent repair capacity. Development of methods for isolating specific viable neurons in quantity (15) should permit detailed quantitative analysis of DNA repair in highly differentiated nerve cells.

REVERSIBLE DIFFERENTIATION OF NEUROBLASTOMA

Neuroblastoma cells are derived from neural crest tissue and, when grown in culture without serum, assume a differentiated state with many of the features of non-dividing neurons (16). This differentiation process can be reversed by the readdition of serum, providing a convenient model system in which response to damage can be assessed quantitatively alongside electrical and enzymatic behaviour in a cloned population.

Differentiated mouse neuroblastoma cells are much more sensitive to UV radiation than proliferating cells when monitored by cloning efficiency in agar. Normal resistance is regained if the differentiated cells are allowed to proceed to the next G1 phase of the cell cycle before irradiation, i.e. radiosensitivity is reversible. In the differentiated mode excision repair is present, but these cells clearly are less capable of immediate, adequate repair than proliferating cells. However, if irradiated,

differentiated cells are allowed to repair damage for a substantial period before being stimulated to replicate DNA by addition of serum, survival increases substantially (17). Being forced into a replication phase right after irradiation appears to be the critical factor in sensitivity of the differentiated cells. Post-replication repair is present in proliferating cells and at certain times in differentiated cells: it has been suggested (18) that the greater sensitivity of the latter may be due to ineffective post-replication repair or to deficiencies in more than one mechanism, with reduction in repair capacity beyond a critical threshold. Response to ionizing radiation has some parallels (James and Kidson, unpublished), while human neuroblastoma cells have somewhat similar behaviour following both forms of irradiation.

Taking the differentiated neuroblastoma cells which have been given adequate time post-irradiation to repair DNA damage slowly, a parallel with neuronal behaviour can be envisaged. Thus, even with low repair capacity, when they are not forced into replication, survival can be maintained.

MUTANTS

The use of *Drosophila melanogaster* mutants is beginning to open up the question of the interrelations between DNA repair systems and mechanisms of genetic recombination. Thus, the *mei-g*^{a} mutant, which reduces meiotic recombination in females, is deficient in the excision of UV-induced pyrimidine dimers in both sexes (19), while other mutants are more selective. Since meiotic exchanges are important in genesis of phenotypic patterns, these findings suggest an essential role in development of repair systems quite apart from that in the organism's coping with DNA damage.

Mutants of *Saccharomyces cerevisiae* which as haploids are sensitive to alkylating agents, UV light and X-radiation, as diploids exhibit elevated frequencies of spontaneous mitotic recombination at the *ade6* locus (2). Clearly in view of the large numbers of mutants available, further work with both organisms will give important insights into the essential roles of at least some repair systems in normal DNA handling. Data so far available for *Dictyostelium discoideum* suggest this organism may be similarly useful, since already some mutants are described in which repair anomalies can be associated with or are distinct from effects on development (21).

Human mutants have already provided circumstantial evidence of the involvement of repair systems in normal development, although so far at a less well-defined level of resolution. This is illustrated particularly by the

correlation of certain mutants with neurological abnormalities, as is evident for some complementation groups of xeroderma pigmentosum (22) and for ataxia telangiectasia (23). Some other heredodegenerative diseases involving the nervous system almost certainly are based on repair defects (22). While it is possible that the nervous system abnormalities seen in these diseases are the result of inadequately repaired chemical or radiation damage, it is also possible that there are deficiencies in normal brain development, especially where there is evidence of prenatally determined mental retardation.

LYMPHOID DIFFERENTIATION

Circulating lymphocytes are very sensitive to ionizing radiation, but stimulation by lectins of DNA replication and morphological transformation is accompanied by increased resistance, which parallels the marked enhancement of DNA repair capacity (24). The available evidence suggests that blast transformation by lectins involves induction of at least one repair system. Studies on lymphocytes from patients with ataxia telangiectasia (25) indicate that the ionizing radiation repair defect is expressed in the lymphoid system and that there is very little increase in the very low repair capacity upon phytohemagglutinin stimulation. In view of the immunodeficiencies seen in ataxia telangiectasia (23), these findings suggest that the underlying repair system(s) may play an important role in lymphocyte differentiation. Since the immunodeficiencies are selective and since there probably are a number of complementation groups, these mutants are promising as a source of information concerning the role(s) of ionizing radiation repair systems in the differentiation of the several immune system functions.

PROTOZOAN NUCLEAR MODIFICATION

Ciliate protozoa contain two types of nuclei, micronuclei and macronuclei. The micronucleus of *Oxytricha* is diploid, divides by mitosis and apparently serves only as a germ line nucleus which undergoes meiosis and intercellular exchange during conjugation. Macronuclei divide amitotically during vegetative growth; they are formed anew from micronuclei following conjugation. This development involves DNA replication and polytenization of chromosomes, the polytene chromosomes subsequently becoming fragmented. There follows extensive DNA degradation and elimination of as much as 97% of the micronuclear sequence complexity (26). The mature macronucleus is formed following a second round of replication, the macronuclear genome consisting of small DNA molecules ranging from 500 to 30,000 base pairs. About 1000 copies of each of the ∿17,000 different DNA molecules are

present in the macronucleus (26). Cells having only macronuclei can grow and divide extensively and it is these nuclei which provide most of the RNA transcription capacity in the organism. Most, perhaps all of these DNA molecules contain the same inverted terminal repeat sequence of approximately 26 base pairs (27).

Here then is an example of a complex processing of DNA for the purposes of differentiation of a protozoon. This processing involves extensive, precise incision steps, with potential recombination between fragments for the purposes of replication. While on present evidence it is not known what enzymes participate in this process, consideration thereof is clearly pertinent in the context of repair in the broadest sense. It will be of interest to see further developments in work on this and other similar organisms, since genome processing is clearly more widespread in differentiation than might have been anticipated.

GENE TRANSPOSITION

Another pertinent area in which exciting advances in knowledge are occurring rapidly concerns gene movement around the genome. Well described in bacteria and their plasmids, the implications for differentiation of complex organisms are considerable. Far from being concerned only with major chromosomal inversions, deletions, duplications or translocations, gene movement could conceivably be a way of switching genes on and off by creating or destroying recognition sites for controlling proteins. Insertion sequence recombinant events can be seen as an extension of repair phenomena in the same way as can genome fragmentation and processing, although the precise enzymologic overlap remains to be determined.

Particularly intriguing at this early stage of our understanding is the evidence for such gene transpositions in the development of immunoglobulin gene functions. Thus, in embryo, variable and constant region DNA sequences of the mouse λ light chain are much further apart than in mature plasmacytes (28). Although somatic rearrangement brings them closer together they are still separated by 1250 nontranslated bases (29), implying a requirement for further RNA processing before or during translation. If such recombinant events are widespread or occur at least for selected gene functions vis à vis differentiation, repair systems concerned therewith become recognizable as critical to the development and stabilization of precise regions of the differentiated genome. Such considerations might conceivably be relevant to such practical problems as high frequency antigenic variation in human and animal parasites.

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THE RELATIONSHIP BETWEEN CELLULAR AGEING AND GENETIC DEFECTS

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ABSTRACT The growth of human diploid fibroblasts through numerous sub-cultures is invariably followed by senescence, degeneration and finally death of the whole cell population. It has been shown that the senescent phase of growth is associated with a number of genetic changes, including a rise in polyploidy and chromosome abnormalities, an increase in somatic mutation frequency, a reduction in repair capacity and a decline in the activity and fidelity of DNA polymerase. It is unlikely that these changes are the result of a gradual accumulation of recessive genetic defects throughout the lifespan since tetraploid fibroblasts have the same longevity as diploid ones. It is more likely that the accumulation of genetic damage during senescence is the final consequence of other changes which occur over a long period of fibroblast growth. One possibility is that spontaneous errors in the synthesis of proteins feedback into the major pathways of macromolecular synthesis, leading finally to a breakdown in the accuracy of information transfer between such molecules. According to this hypothesis, genetic damage can be a major cause of cell death, but it is not the underlying cause of the process of ageing.

INTRODUCTION

A large number of foetal or adult human fibroblast strains have been routinely grown in many laboratories and on every occasion the total population of cells has died out after many subcultures. There are no reports of permanent cell lines arising from such cultures. The lifespan of human fibroblasts was assigned to three phases by Hayflick & Moorhead (1). Phase I is the establishment from tissue of the primary culture; Phase II is the long period of growth during which the cells retain their normal morphology, and Phase III is a period of slower and slower growth during which the cells become morphologically abnormal and finally cease to divide. The lifespan of populations of human fibroblasts is very

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variable both between strains and within one strain. For example, the range for foetal lung strains MRC-5 and WI-38 is 55-75, and 38-60 population doublings respectively (2). It is also known that clones of cells have very different growth potential (3). These results by themselves strongly indicate that stochastic or random events are important in determining the lifespan of any one culture.

In any discussion of the ageing of these cells, we have to consider the relationship between the intrinsic events occurring during Phase II, which in some way accumulate with cell division, and the overt changes in Phase III which finally lead to cell death. In this review, I will concentrate on the possible significance of various changes at the DNA level, and particularly on results obtained at the Mill Hill laboratories with strain MRC-5.

RESULTS

Chromosome Changes It was discovered many years ago that human cells retained their diploid karyotype through numerous cell generations. Although aneuploids, tetraploids and chromosome abnormalities occur in normal cultures, their frequency remains more or less constant throughout Phase II. As the end of the lifespan is approached, there is a striking increase in each of these types of chromosome alteration (4,5,6,7). Nevertheless, in the very oldest cultures which have been examined, i.e. the very last dividing cells, at least 50% of the cells seem to have a normal diploid karyotype. This result shows that gross chromosome changes are not in themselves a likely cause of cell death.

It is very important to examine the affect of ploidy on longevity. This has now been done in two laboratories by different methods and the results are the same. Hoehn et al (8) fused young diploid skin fibroblasts and recovered tetraploid hybrid clones. The lifespan of these was not significantly different from that of diploids. Thompson and Holliday (9) treated MRC-5 cells with colchicine and obtained a high proportion of tetraploid cells among the survivors. On further subculture until death, the proportion of tetraploids remained constant, which indicates that both the growth rate and longevity of diploids and tetraploids is similar. These results have important theoretical implications for the somatic mutation theory of ageing, which will be discussed later.

Gene Mutation It is now possible to measure with some accuracy the frequency or rate of gene mutation in human fibroblasts, for example, mutation to resistance to 6 thioguanine (TG) or 8 azaguanine (AG). Since this involves

growing colonies, it is obvious that many cell generations must elapse from the time of the mutation to the time of scoring. The method is not therefore applicable to senescent cells, which have very limited growth potential. To circumvent this difficulty, Fulder and Holliday (10) applied the procedure of Wajntal and DeMars (11) which involves staining individual cells for altered glucose-6-phosphate dehydrogenase activity. They detected spontaneous cell variants which had increased amounts of the enzyme, presumably as a result of changes in the regulation of enzyme synthesis. The phenotype is heritable and the number of heavily stained cells could be increased by treatment with standard mutagens. In several experiments, the frequency of spontaneous variants increased throughout the lifespan of MRC-5. There was a slow but significant rise in frequency during Phase II and a very rapid increase during Phase III. The kinetics very strongly suggest that there may be an exponential increase in observed mutation frequency, particularly if account is taken of the decreasing number of dividing cells in Phase III (10,12). If this is so, then the rate of mutation is increasing throughout the lifespan, provided that the variants are not growing faster, or have a longer lifespan than normal cells. It would be of great interest to measure the rate of mutation to AG or TG resistance at different times during Phase II, but this experiment has not yet been done.

DNA Synthesis It has been known for many years that the proportion of cells entering S phase declines during in vitro growth and ageing, but the extent of this effect is controversial. Cristofalo and Sharf (13) believed that they could determine the age of a culture by measuring the ^{3}H thymidine labelling index, but Maciero Cuelho (14) maintains that almost all cells eventually enter S phase, even in late passage cultures. More recent results of Vincent and Huang (15) tend to support the latter conclusion, since it was found that many human cell strains retained a high labelling index until late in their lifespan. There was subsequently a precipitous drop in the number of cells entering S phase.

Using young and old cultures of MRC-5, Petes et al (16) measured the rate of replicon elongation by fibre autoradiography. They found that senescent cells replicated their DNA about 25% more slowly than did young cells. The distribution and length of replicons was not affected. Linn et al (17) found that the activity of DNA polymerase was extremely low in passage 58 MRC-5 cells and at passage 62 was barely detectable, even though this particular culture grew until passage 70.

Fidelity of DNA Polymerase Linn et al (17) measured the fidelity of DNA polymerase (α form) isolated from passage 19, 28,58 and 62 MRC-5 cultures. They used DNA templates containing just two complementary bases and compared the incorporation of homologous and non-homologous labelled nucleotides. Most of these experiments were carried out with Mn^{++} as essential cation, since with Mg^{++} the error levels were usually too low to measure. Using a wide variety of templates and nucleotides, they found a consistently higher level of misincorporation when enzyme from senescent cells was used. The decline in fidelity with age has been confirmed by V.M. Murray (personal communication), who was able to reduce background counts to a level which enabled him to measure misincorporation frequencies in the presence of Mg^{++}.

DNA Repair Initial reports indicated that repair mechanisms in senescent fibroblasts were not impaired (18,19), but subsequent studies have not confirmed these results. Hart and Setlow (20) measured unscheduled DNA synthesis after UV light during in vitro ageing of WI-38 and related it to the proportion of cells capable of normal DNA synthesis. Both the number of cells with unscheduled DNA synthesis and the extent of this synthesis declined during Phase III growth. Mattern & Cerutti (21) measured the excision of damaged thymine from DNA after γ-radiation and found that senescent cells were unable to carry out this repair. Epstein et al (22) report that the rejoining of single strain breaks after X-ray treatment is less efficient in senescent cells, contrary to the earlier results of Clarkson and Painter (19).

The sensitivity of human fibroblasts of different age to ionising radiation has been measured by Cox and Masson (23). It is not possible to use senescent cultures in these experiments, but passage 45 cells were shown to be slightly more radiation resistant than early passage cells. This strongly indicates that late Phase II cultures have not accumulated a significant number of genetic lesions.

It is important to measure carefully the lifespan of fibroblasts with repair deficiencies. Since there is intrinsic variation in longevity of parallel cultures of the same strain, it is necessary to use several cultures to obtain a reliable measure of lifespan (just as it is necessary to use many animals to measure with accuracy their average lifespan). K.V.A. Thompson (personal communication) passaged four Fanconi's anaemia cultures from one individual and found that the average lifespan was 29 passages, whereas control cultures from donors of similar age all exceeded 50 passages. Comparable experiments with Bloom's syndrome and Ataxia telangiectasia have not been published, but from unpublished

reports it seems likely that their lifespan is significantly shorter than that of control cells. On the other hand Xeroderma pigmentation cells may have a normal lifespan (15, 24).

Viral Probes Another approach to the study of genetic changes during ageing is to infect senescent cells with normal virus particles and then screen the progeny for mutations. Holland et al (25) used Polio virus and measured the mutation frequency to guanidine resistance amongst populations grown in young or senescent WI-38. They could detect no difference in mutation frequency. Fulder (26) carried out similar experiments with a much larger virus, Herpes simplex type 1. He used three temperature sensitive mutants and measured by fluctuation tests their rate of reversion to wild type. One mutant (ts E) reverted at a higher frequency when grown in senescent MRC-5 cells than when grown in young cells; another (ts G) reverted at lower frequency, and the third (ts D) showed no significant difference. It is possible that ts E and ts G are reverted through quite different pathways of mutation, which alter in different ways during ageing. For instance, "error prone" repair may decline, but as we have seen, the fidelity of DNA synthesis also declines. In any event, these experiments show that the situation is more complex than that indicated by the results with Polio virus, and that further studies are needed.

DISCUSSION

I will first consider the possibility that *in vitro* ageing is due to the gradual accumulation of recessive mutations of one kind or another, to a point at which growth is no longer possible. At the outset, it is important to note that although the cells are diploid, about 5% of the active genome (the X chromosome) is haploid. The argument is that recessive mutations initially have no effect, but that as they accumulate, the chance that both copies of an essential gene will be inactivated becomes greater and greater. T.B.L. Kirkwood (personal communication) has made the following calculation. Assume there are 10^3 or 10^4 indispensible genes for normal growth and that the lifespan is about 10^2 generations. If 99.99% of the cells are to receive two hits (mutations) in one pair of homologous genes, the spontaneous mutation rate would have to be 7.5×10^{-3} or 3.0×10^{-3} per gene/cell generation (for 10^3 or 10^4 indispensible genes, respectively). These are absurdly high rates. Moreover, if either was true, then 1-3 mutations would occur in every generation on the X chromosome, assuming it contains 10^2 or

10^3 indispensible genes. Since these genes are essential for growth, it is clear that high cell viability would be inpossible from the outset. An even more powerful argument is provided by the experimental demonstration that tetraploid fibroblasts have the same longevity as diploid ones. Tetraploids must be able to accumulate many more recessive mutations before any are expressed.

Suppose mutations are much less common, but often have dominant deletarious effects. In this case, it is necessary to take into account the effect of cellular selection, since every time a daughter cell inherits a dominant mutation, it will, by definition, grow more slowly and therefore be selected against. In these circumstances, cells will always be left which are free of such mutations and the population would increase indefinitely.

There remains the possibility that mutations initially have little phenotypic effect i.e. they are recessive, but as significant numbers of them accumulate, complex metabolic interactions occur which are increasingly deletarious (Fulder (12) refers to this as the 'mutation catastrophe' theory of ageing). The mutants would become dominant or codominant only when many different ones exist in a cell. This still requires a high mutation rate, since every single late passage cell must contain many of those hypothetical genetic changes. Tetraploid fibroblasts would accumulate twice as many mutations per cell generation, so to explain their unaltered lifespan, this would have to be compensated by a two fold reduction in the overall deletarious effect of each mutation. This seems extremely unlikely. Finally, any mutation theory of ageing would predict that cells become more and more sensitive to radiation with cumulative cell divisions, but this prediction is not borne out (23). The overall evidence strongly indicates that the intrinsic changes occurring during Phase II growth are not due to the accumulation of genetic lesions.

Almost all the experimental results show that the phenotype of fibroblasts remains outwardly normal during the long period of Phase II growth, and that a variety of genetic changes occur at an increasing rate during Phase III. This appears to be true for abnormalities in karyotype, mutations, DNA synthesis and repair. If ageing is "programmed", then it must be concluded that after a specified number of cell divisions, the normal controls on the maintenance of the integrity of the genetic material are relaxed. Repair mechanisms might be switched off; the factors ensuring fidelity of DNA synthesis may be altered, and DNA polymerase may finally disappear. The evidence that alterations in the genetic apparatus of one kind or another are the actual cause

of cell death is reasonably convincing, but this conclusion in itself does not help to explain the intrinsic changes occurring throughout Phase II.

The protein error theory (27) provides a possible basis for these changes, as well as those in Phase III. A proportion of spontaneous errors in protein synthesis would be expected to affect the accuracy of transcription or translation, and this feed-back could lead to an increase in error level with every cell division. This increase could be exponential and irreversible, but it might take 50 or more divisions before any change in phenotype was seen. Changes in enzymes will eventually affect the accuracy of DNA synthesis and the efficiency of repair, and this would lead to mutations and chromosome abnormalities. The evidence suggests that mutations may accumulate exponentially throughout the lifespan of fibroblasts (10,12). The decreased longevity of Fanconi's anaemia fibroblasts, or those with other genetic defects (28,29) could be due to an elevated level or transcriptional or translational errors. Agents which might be expected to increase such errors, such as 5FU, BUdR or Mn^{++}, are known to shorten the lifespan of MRC-5 (30, and unpublished observations).

Apart from DNA polymerase, a number of metabolic enzymes have been shown to be altered in senescent fibroblasts. These include glucose-6-phosphate dehydrogenase (30), 6-phosphogluconate dehydrogenase (30), lactic dehydrogenase (31) and hypoxanthine phosphoribosyl transferase (32). On the other hand, changes were not discernible in phosphoglucose isomerase (33). So far, no one has succeeded in measuring the accuracy of protein synthesis in fibroblasts, and until this is done, it will not be possible to test directly the protein error theory of ageing.

It was mentioned at the outset that the lifespan of human fibroblasts is intrinsically variable and that stochastic events are likely to be involved in determining longevity. A basis for this is provided by the commitment theory of cellular ageing (2). This states that cells are initially immortal, but that there is a fairly high probability that committed cells with a finite lifespan will be produced during division. The final loss by dilution of immortal cells is a stochastic process, which determines the eventual culture lifespan. In terms of the error theory, committment to senescence is the beginning of the irreversible build up of errors in macromolecules, which will after many generations lead to the extinction of the population.

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THE ACUTE AND CHRONIC EFFECTS OF ULTRAVIOLET ENERGY ON MAMMALIAN SKIN[1]

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ABSTRACT Ultraviolet energy has profound influences on the structure and function of the epidermal and dermal components of mammalian skin. These changes may eventuate in cutaneous epidermal cancer formation. Among the acute UV responses DNA damage and repair aspects have received a significant amount of attention as potentially relating to carcinogenesis. This relationship has been emphasized by the discovery of DNA repair defects in patients with XP, a disease in which sunlight induced skin cancers occur within the first few years of life. Chronic repeated UV exposures result in striking epidermal and dermal alterations. The epidermal changes include increased cell production and decreased cell transit time. Eventually the production of abnormal cells occurs. In the dermis the production of abnormal connective tissue and mucopolysaccharide elements is prominent. This may be due, at least in part, to UV induced alteration in dermal fibrocytes. It would appear likely that the UV effects on both the epidermis and dermis lead to epidermal carcinogenesis.

INTRODUCTION

Cutaneous epidermal cancers, basal cell epitheliomas and squamous cell carcinomas are by far the most common of all human malignancies. The primary stimuli for the formation of these tumors appears to be the ultraviolet rays (UV) from the sun. Experimental studies in mice suggest that UV carcinogenesis starts with the first exposure (1,2). However a definitive correlation of any specific acute UV effects with cancer formation has not been accomplished as yet.

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ACUTE EFFECTS

The sunburn reaction is indeed the most common adverse acute effect produced by sunlight energy. Though the delayed erythema response is the most visible the effects of these rays on epidermal macromolecules and mitoses is more likely to be related to carcinogenesis. Inhibition of DNA, RNA and protein synthesis and mitosis formation occurs within 1 hour post UVB (290-320 nm) irradiation (3-5). By 24 hours recovery occurs followed by acceleration of all of these functions associated with epidermal hyperplasia. This hyperactivity and hyperplasia gradually subsides, though increased mitotic activity has been demonstrated for up to 40 days following a single exposure (6).

A relationship between acute DNA damage and carcinogenesis has been suggested. Pyrimidine dimer formation and repair by photoreactivation (7), postreplication (8), and excision mechanisms (9), have been described in a variety of mammalian cell types. The excision repair process has received the greatest emphasis in epidermal UV injury to date. Thymine dimerization has been induced in tissue culture cells by sunlight as well as artificially produced UV rays. This injury and repair has been demonstrated in the epidermis and in vivo as well as in cell culture systems (8,10). The possible relationship to cancer formation has been emphasized by the discovery of a defect in this repair enzyme system in patients with xeroderma pigmentosum (XP) (11,12). The primary photosensitive problem in XP is an inordinate sensitivity to the carcinogenic effects of sunlight. It seems likely that this problem relates to their DNA repair defects.

CHRONIC EFFECTS

In human skin chronic sun exposure results in a characteristic weather beaten, leathery appearance frequently associated with actinic keratoses and at times cancer formation, especially in light complexioned individuals. Using the hairless mouse model and repeated UV exposures it has been possible to examine the progressive epidermal changes induced by such chronic injury ranging from benign hyperplasia to premalignancy and frank invasive squamous cell cancer (13). Examination of the epidermal kinetics revealed a progressive increase in mitoses and premitotic DNA synthesis ("S") in the germinative cells with a shortening of the G^2 and "S" phase. In addition, there was a progressive reduction of the transit time from the basal layer into the stratum corneum. Cell production was greater than cell loss resulting in epidermal hyperplasia. When malignancy and invasion occurred it appeared

to develop from the basal cells. However as the tumors grew and became more anaplastic the whole cell mass became abnormal and the germinative cells were no longer identifiable. Profound dermal changes associated with chronic sun exposure occur in human skin. Progressive degeneration occurs in the upper dermis. Specific findings include dilated blood vessels, the accumulation of acid mucopolysaccharides and abnormal appearing fibrocytes, the loss of mature collagen (but an increase in the soluble component) and a marked increase and degeneration in elastic tissue referred to as actinic elastosis (14-17). Actinic elastosis is the most prominent and obvious dermal change due to chronic solar damage. The action spectrum for the experimental induction of elastosis falls in the UVB range. The mechanism may relate to fibrocyte damage and altered function.

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DEATH OF *ESCHERICHIA COLI* CELLS AS A CONSEQUENCE OF DAMAGE TO DNA BY FAR-ULTRAVIOLET RADIATION[1]

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ABSTRACT This paper deals with unanswered questions concerning death of *Escherichia coli* cells after far-ultraviolet (UV) irradiation. Attention is called to poor correlations between DNA repair and survival in repair-competent cells given various pre- and post-UV treatments. Under some of these and other conditions, a large percentage of pyrimidine dimers remain unexcised. The evidence is reviewed concerning UV induction of irreversible metabolic responses associated with cell death. Since cyclic 3',5'-adenosine monophosphate (cAMP) and its receptor protein regulate cessation of respiration and cell killing, but have no known regulatory roles in DNA repair and DNA replication, death may be the result of induced biochemical and physiological responses that become irreversible.

One of the most obvious and important consequences of radiation damage to DNA is death of the cell. Yet, in spite of extensive study of the effects of far-ultraviolet radiation (UV) on the bacterium *Escherichia coli* during the past 50 years, the causes of radiation cell death remain poorly understood. Since the discovery of DNA repair most theories of UV radiation cell death in *E. coli* have involved dysfunctional DNA caused by insufficiencies in repair processes (1). Contributing to and strongly supporting this point of view is the fact that radiation-sensitive mutants of *E. coli* are usually deficient in some step or pathway in DNA repair. In this paper are considered some of the unanswered questions about the relationships among DNA repair, physiological consequences of radiation-damaged DNA, and death of repair-competent *E. coli* cells.

In most *in vivo* experiments on DNA repair, mutant strains are studied and the results are compared to those of wild-type repair-competent cells. The experimental conditions are usually such that the control cells upon incubation after irradiation show evidence of complete or nearly complete repair as

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indicated by the removal of dimers or return of DNA to its normal size. Even under these conditions survival is often less than 100%.

In another type of experiment, repair and survival are measured in repair-competent or repair-deficient cells given pre- or postirradiation treatments that cause increases or decreases in survival. In most cases poor correlations have been found between DNA repair and survival (see ref. 1 for review). More recently it was shown that no effect on excision of dimers was brought about in repair-competent cells by post-UV treatments of cyclic AMP and the protease inhibitor antipain (2), each of which decreases viability of the culture. Also liquid-holding recovery (LHR) treatment (incubation in buffer), long thought to bring about increased survival by allowing time for more complete excision repair, causes a decrease in rate and extent of excision of dimers in an *E. coli* K12 *uvr*$^+$ *rec*A$^+$ strain (3). In K12 strains LHR does not take place in *rec*A$^+$ strains (4). Similar experiments performed on an *E. coli* B/r strain after a relatively high fluence giving less than 10^{-5} survival, showed a parallel between viability increase and excision of dimers, but the maximum extent of excision was 24% (5). In neither case were the cells transferred from buffer to complete medium to see what effect that change would have on excision.

Poor correlations between survival and repair are related to another vexing question. What is the significance of incomplete excision of dimers under various conditions such as growth on glycerol (60% excision) (6) and preirradiation starvation for tryptophane and thymine (essentially no excision) (7)? Since excision was measured on a culture containing both live and dead cells, it was possible that the remaining dimers were in the dead cells. If so, one would expect the replicated DNA of the culture starved prior to irradiation to be free of dimers. However, dimers were found in both replicated and unreplicated DNA (8). We developed a method for separating pure populations of living and dead cells from cultures incubated for 90 min after irradiation of the glycerol-grown cells (9) and found that the same percentage of dimers (60%) were excised from the DNA of each population (6). When the live cell population was reirradiated, dimers were immediately excised, showing that the excision system was not inactive, but seemed to be ignoring the residual dimers.

Other experiments show that dimers are found in the daughter strands of irradiated *uvr*$^-$ cells (10) and it has been proposed (10) that the dimers are gradually diluted out in the DNA of succeeding generations of surviving cells. Postreplication repair and dilution may aid in toleration of dimers in repair-competent cells as well, but why should dimers remain when the capability for excision is present? Another question

is: why are a small number of dimers lethal to *uvr*$^-$ cells when a greater number of unexcised dimers can be tolerated by the survivors in a *uvr*$^+$ culture (11). The possibility of selective excision of critical dimers as well as accurate dimer bypass mechanisms should be explored.

The results of photoreactivation and many other experiments leave practically no doubt that the formation of pyrimidine dimers in DNA of a UV-irradiated *E. coli* cell ultimately results in death of the cell. For this reason most work on irradiated cells has been concentrated on DNA repair and DNA synthesis. However, certain physiological changes also occur after irradiation and these are closely associated with death and seem to be the result of damage to DNA. An important one is cessation of respiration about 60 min after UV (12). Those cells that stop respiring do so irreversibly and they are dead (9,13). The process is dependent upon RNA and protein synthesis (12) and requires the *rec*A$^+$ and *lex*A$^+$ gene products (14). Like other rec/lex responses (1,15), cessation of respiration in *uvr*$^-$ strains requires only about 1/10 the fluence of UV to produce the same effect seen in wild-type cells.

Complete loss of pyridine nucleotides from irradiated cells is also associated with cessation of respiration and cell death (16). The loss, beginning about 30 min after UV is selective and unaltered pyridine nucleotides, NAD^+ and $NADP^+$ are found in the suspending medium (17). Pyridine nucleotides are not lost by irradiated cells if respiration is maintained by certain pre- or postirradiation treatments (1) or if the *exr*A$^-$ (*lex*A$^-$) gene is present (16).

The requirement of RNA and protein synthesis and *rec*A$^+$ and *lex*A$^+$ gene products for cessation of respiration suggests that an operon is being induced by UV as in the case of the lambda lysogen. Recently we reported that cessation of respiration and killing after UV radiation is regulated by cyclic 3',5'-adenosine monophosphate (cAMP) and its receptor protein (18,19). When exogenous cAMP is added to an irradiated wild-type culture, respiration is shut off more completely and more cells die than when it is absent (18,19). Strains unable to synthesize cAMP because of a deletion of the gene for adenylate cyclase (Δ cya) or which are deficient in the cAMP receptor protein (CRP), (*crp*$^-$), do not shut off respiration after UV; however, when cAMP is added to the Δ cya strain, their respiration is shut off and more cells are killed; cAMP does not affect these responses in the *crp*$^-$ strain (19). The cAMP-CRP complex is necessary for transcription of inducible operons in *E. coli* and this is its only known function (20). Cells grown on glycerol have higher intracellular levels of cAMP than when grown on glucose (21) and it is significant that both respiration and viability are more sensitive to UV

when cells are grown on glycerol (12,16).

Involvement of the *recA*$^+$, *lexA*$^+$ and the *cya*$^+$ and *crp*$^+$ gene products provides the only genetic evidence, albeit indirect, that shutting off of respiration involves induction of an operon by UV as in the case of the lambda lysogen. Nevertheless, the involvement of these gene products coupled with a requirement for RNA and protein synthesis raises questions about the relationships among cessation of respiration, DNA repair and cell death.

An important question is: Do the cells die because they stop respiring or do they stop respiring because they are dead? After very large fluences of UV, respiration is not shut off probably because no transcription occurs. At lower fluences the timing of events does not help in resolving the problem since the first indications of cessation of respiration and cell death are both seen about 40 min after UV as indicated by the beginning of lysis of cell membranes by Triton X-100 (13). Only the membranes of those cells that are not respiring and are dead (as measured by colony-forming ability) are vulnerable to the detergent (9,13). Cessation of respiration may be the final manifestation of an induced process that begins somewhat earlier and involves a sequence of biochemical changes. If a double-strand break or failure to reinitiate DNA synthesis were a lethal event and if only dead cells could be induced, it is difficult to see how the cAMP-CRP complex would function to amplify shutoff of respiration and killing when cAMP is added to an irradiated culture. The complex might regulate DNA repair or replication and thus influence whether a cell dies and shuts off its respiration. However, there is no evidence for regulation of these functions by cAMP.

Another possibility is that induction of the sequence of biochemical changes occurs in irradiated cells before any are dead and that only when the induced biochemical changes becomes irreversible, as in those that cease respiring, are they dead. In this situation cAMP and its receptor protein could regulate cessation of respiration and cell killing and pre- and postirradiation treatments that alter viability might act at various places in the induction system, e.g., by influencing cAMP levels, as in glucose vs. glycerol grown cells and in production of a fradulent messenger RNA, as has been proposed for the action of 5-fluorouracil (1).

What role would DNA repair play in radiation cell death of repair-competent cells if induction of irreversible biochemical and physiological changes were responsible for death? The formation of pyrimidine dimers would doubtlessly be the initial inducing event and these inducing lesions might be repaired even after the cell has passed the point when biochemical and physiological changes are irreversible. Perhaps,

as is indicated by our excision experiments (6), cells destined to live and die carry out essential excision of dimers equally well. However, since the dead cells lose pyridine nucleotides it is to be expected that there would be differences in the repair of single-strand breaks in DNA brought about by the incision process. We have presented evidence (22) that more single-strand breaks in DNA are repaired in 5-fluorouracil-treated cells, where respiration is maintained and viability is markedly increased, than in untreated cells. Evidence of this repair is first seen 60 min after UV. Excision of dimers is not affected by the 5-fluorouracil treatment. Repair of UV damage seems to be very accurate in repair-competent cells as evidenced by the small number of mutations produced compared to the number of photoproducts formed.

In conclusion, it is evident there are many questions remaining to be answered concerning the way UV radiation kills an *E. coli* cell. More experiments are needed in which various types of DNA repair are followed in repair-competent cells given pre- or postirradiation treatments that influence viability in a significant way. The location of residual dimers in the DNA of live cells and dead cells should be examined. It is also important that the biochemistry of radiation cell death be studied to determine the nature of that event or process.

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ACTION OF THREE MUTAGENS ON HeLa DNA REPLICATION[1]

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Three different mutagens, 4-nitroquinoline-1-oxide (4NQO), adriamycin (Adr), and ethylenimine (EI), were examined for their effects of DNA replication in HeLa cells using velocity sedimentation techniques with alkaline sucrose gradients.

At a dose of 2×10^{-7} M, where DNA synthesis is inhibited by about 50% within one hour after treatment, 4NQO does not cause detectable breaks in parental DNA. Although 4NQO is generally considered to be "UV-like", because it induces long patch repair (1) and is not excised by repair-deficient (xeroderma pigmentosum) human cells (2), its earliest and most striking effect on DNA synthesis is, like x-rays, to inhibit replicon initiation. A second effect of 4NQO, appearing at later times, inhibits chain elongation (submitted to Cancer Research).

Adriamycin (Adr) intercalates into DNA and, although it is a known mutagen (3), it does not induce detectable repair replication even at doses that almost abolish semiconservative DNA replication (4). These properties suggest that it does not form a covalently bound adduct or induce strand breaks and that its mode of inhibition of DNA synthesis would not include an effect on initiation.

Nevertheless, Adr does inhibit replicon initiation, although this effect on DNA synthesis is a minor one, with the major one being inhibition of chain elongation rate (submitted to Cancer Research).

At a concentration ethylenimine (EI) that inhibits DNA synthesis by about 50 percent (5×10^{-4} M), this chemical induces lesions in parental DNA that result in strand breaks when the DNA is exposed to alkali (alkali sensitive bonds). The inhibition of DNA synthesis is caused both by a strong block to replicon initiation and the inability of nascent DNA to extend past the lesions in parental DNA (Figure 1).

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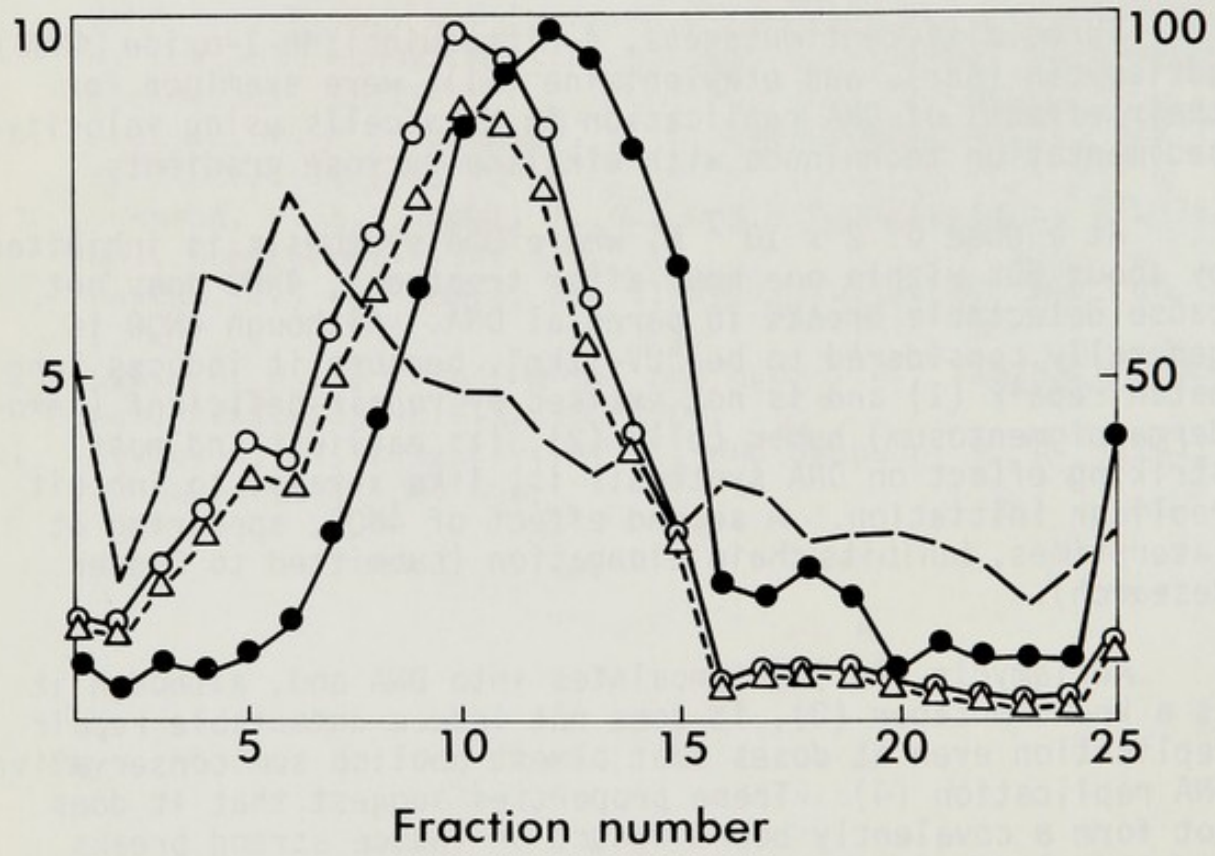

FIGURE 1. Effect of ethylenimine (10^{-4} M) on parental DNA and on nascent DNA in HeLa cells. Alkaline sucrose gradient profiles from cells prelabeled for 24 hr with ^{14}C-thymidine and pulse labeled for 10 minutes with ^{3}H-thymidine immediately after a 30 minute incubation with drug. Open circles, ^{3}H- radioactivity, plotted as % of fraction with highest counts (fraction 10). Open triangles, same ^{3}H data, but plotted as % of total ^{3}H in control cells. Closed circles, ^{14}C radioactivity, plotted as % of fraction with highest counts (fraction 12). Broken line without data points is the plot of % total ^{3}H radioactivity in control cells. ^{14}C radioactivity in control cells was primarily at bottom of gradient (data not shown). Sedimentation from left to right.

Two hrs after exposure to lower concentrations (10^{-4} M) of EI the nascent DNA does extend past the parental lesions; the overall rate of DNA synthesis increases at the same time (submitted to Cancer Research).

These studies show that three DNA damaging drugs of widely varying action all inhibit replicon initiation, although to different relative extents. These observations suggest therefore that inhibition of replicon initiation is a very common response of human cells to DNA damage and reinforce our previous proposals (5-7) that a large subunit of chromosomal DNA with molecular weight of about 10^9 is involved in the regulation of mammalian DNA replication. In this scheme, the super-supercoiled structural subunit of Cook and Brazell (8) is the same as the subunit for replication known as the replicon cluster. A single "hit" (i.e., effective lesion) within this subunit changes its conformation (relaxes the supercoil). Supercoiling is required for replication of λ phage DNA and Col E_1 plasmid DNA in Esherichia coli (9) and presumably for replication of mammalian DNA as well. The altered conformation after damage then would prevent the attachment of those enzymes (and perhaps other proteins) required to initiate replication at the many origins of replicons within the cluster. Initiation of replication of this entire subunit then would be prevented until the lesion within it is repaired and its original conformation is restored.

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UV IRRADIATION AND THE MAMMALIAN CELL CYCLE[1]

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ABSTRACT The efficiency of cyclobutane pyrimidine dimer production by UV varies through the cell cycle as predicted by a computer model based on changes in target geometry. But changes in UV sensitivity are not entirely due to the changes in damage; other factors influence cell survival, probably including DNA precursor pool sizes.

In assessing the variations in sensitivity of cells to UV, and their capacity to repair, through the cell cycle, results should be related not to the UV dose, but to the damage done, since the effectiveness of UV in damaging DNA may vary during the cell cycle.

DNA Damage Related to UV Dose. Figure 1a demonstrates two approaches to this problem; experimental, measuring the efficiency of thymine-thymine dimer (T^T) formation, and theoretical, with a computer model to simulate the effects of variations in target geometry on UV absorption. The agreement between the two curves indicates that changes in chromatin geometry during the cell cycle may fully account for the variation in the amount of DNA damage (1).

We can now plot survival data for HeLa in terms of UV dose or T^T formation (Figure 1b). A maximum D_0 in mitosis in terms of UV dose is converted to a minimum D_0 in terms of dimer damage, with the peak of D_0 now occurring in S phase (1). Similar results have been obtained with CHO-K1 cells. There is a clear resemblance between the cell cycle survival curves for UV damage and for X-ray damage.

The fluctuation in UV sensitivity through the cell cycle when plotted against dimer damage suggests that survival is a function of the cell's varying ability to repair the dam-

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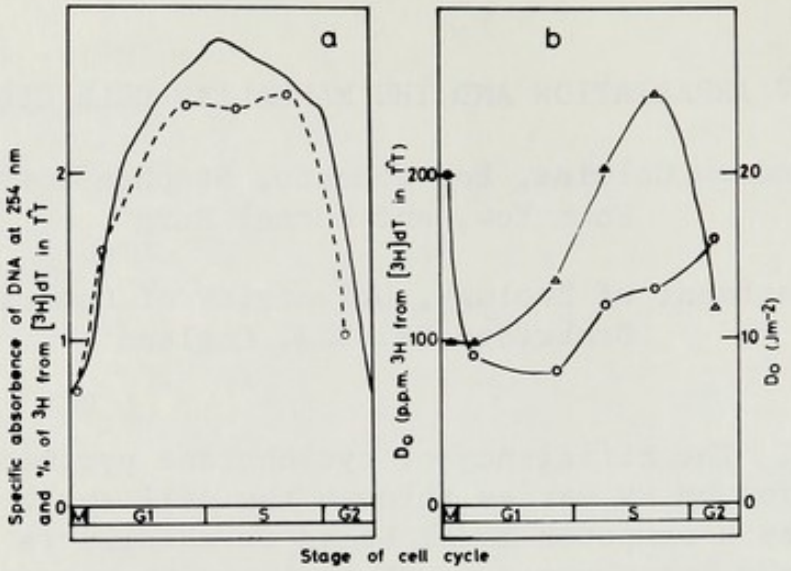

FIGURE 1. UV damage and survival through the HeLa cell cycle. (a) Solid line; computer prediction of specific DNA absorbence at 254 nm. Broken line; yield of T^T dimer from 1440 Jm^{-2} UV. (b) UV survival; D_0 in terms of UV dose (o) or T^T dimers (Δ).

age. Before discussing possible influences on repair capacity a digression into details of experimental interference in repair is necessary.

Uncoupling Repair. UV repair can be regarded as two processes; recognition/incision/excision, and DNA synthesis/ligation. Inhibitors of DNA synthesis (hydroxyurea (HU), 1-β-D-arabinofuranosylcytosine (ara C), etc.) block the second process but not (as far as is known) the first. Repair is thus "uncoupled", and breaks accumulate in pre-existing DNA, as is shown by these effects: (i) a much increased rate of denaturation of DNA in alkali (2,3); (ii) a much decreased rate of sedimentation of DNA in alkaline sucrose gradients (2-4); (iii) a block in the restoration of DNA supercoiling in nucleoids of irradiated lymphocytes (5); (iv) an increased binding of fragmented DNA to nitrocellulose (3); (v) a gross decondensation of metaphase and interphase chromosomes seen after fixation (3,4,6); (vi) a reduction in cell survival following brief treatment with inhibitor (6,7). These effects are rapidly reversed when the inhibitor is neutralised (by deoxyribonucleosides dA, dC, dG and dT in the case of HU, or by dC alone in the case of ara C; i.e. repair synthesis restarts and breaks are rejoined (3).

Ara C, although it may be incorporated into DNA, is thought to inhibit DNA polymerase. It has maximal uncoupling effect at 10^{-5}M in mitotic and G_1 HeLa cells; at this concentration the inhibition is not affected by competition from

the endogenous deoxyribonucleoside pool. HU inhibits DNA synthesis less directly, acting on ribonucleotide reductase and depleting DNA precursors. The effect of HU on repair should be more pronounced in cells (or at stages of the cell cycle) having small DNA precursor pools. This is confirmed by the effect of HU on the survival of CHO-K1 cells irradiated at various stages of the cell cycle (7).

Despite this and other evidence (8,9) that agents which inhibit replicative DNA synthesis also inhibit repair, many workers continue to use HU in the belief that replicative synthesis will be selectively suppressed.

Is the Availability of DNA Precursors a Rate-Limiting Factor in Repair? We have shown that repair can be uncoupled by artificially limiting DNA precursor production (with HU), or by blocking DNA polymerase (with ara C). As to whether

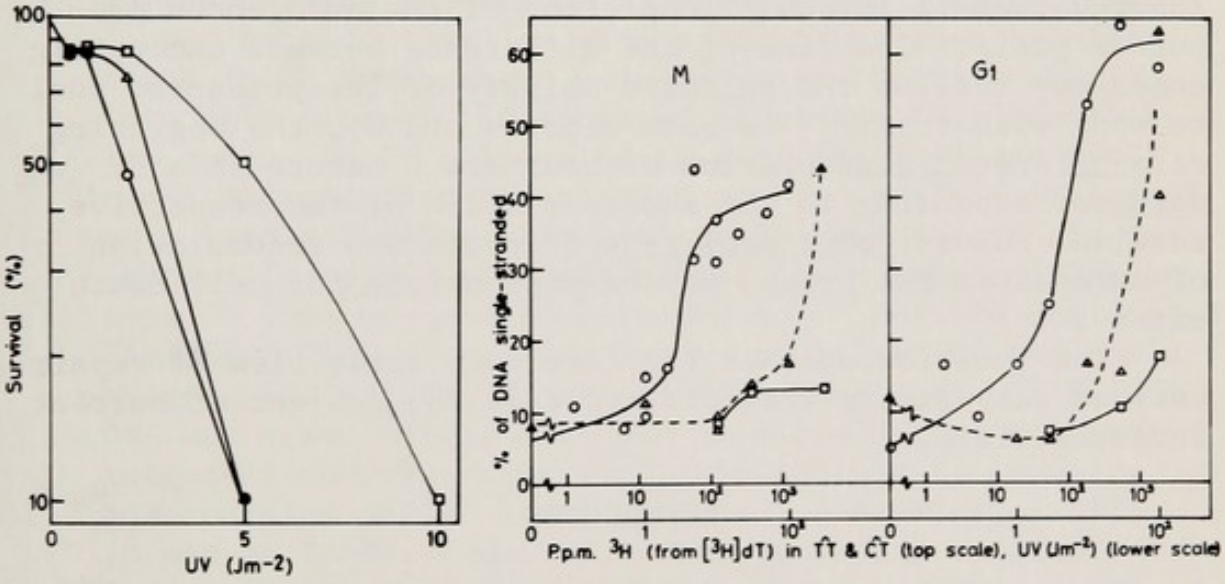

FIGURE 2. Survival of T lymphocytes. After UV, cells were incubated for 3 days in MEM + 20% dialysed serum (○) or in this medium with dA, dC, dG and dT at 10^{-5}M (△) or 10^{-4}M(□) each. Survival was scored by trypan blue exclusion.

FIGURE 3. Uncoupled repair in synchronised HeLa cells. [^{3}H] dT-labelled HeLa cells in mitosis or G_1 (4) were irradiated with UV and incubated for 30 min in MEM + 6% dialysed serum (--△--), or in this medium with ara C at 10^{-5}M (—○—), or with dA, dC, dG, dT at 10^{-4}M each (—□—). After lysis in alkali for 15 min, the extent of denaturation of DNA was estimated by hydroxyapatite chromatography (2,3). Results are plotted against UV dose and corresponding dimer damage.

normal cellular repair may be uncoupled, we have several lines of evidence:

(a) Human T lymphocytes have very small DNA precursor pools (10) and are very sensitive to UV (D_0 = 1.5 Jm^{-2} in medium with dialysed serum). This sensitivity is reduced by added DNA precursors (Figure 2).

(b) A large dose of UV (240 Jm^{-2}),with no inhibitor, decondenses mitotic HeLa chromosomes; the effect is much reduced if dA, dC, dG and dT are provided in the incubation, suggesting that repair of severe UV damage exhausts the endogenous pool (3).

(c) When mitotic or early G_1 HeLa cells are irradiated and incubated, 10^{-5}M ara C induces uncoupling (monitored by alkaline denaturation of DNA) at low UV doses; without ara C, uncoupling occurs at much higher doses, and can be largely suppressed by dA, dC, dG, and dT (10^{-4}M each) (Figure 3), showing that it is due to depletion of the endogenous precursor pools. The size of the difference between uncoupling doses may reflect the relative ability of the precursor pool to cope with repair. In both mitosis and G_1, the beginning of accelerated denaturation without ara C occurs at a UV dose corresponding to the shoulder width of the respective survival curves; thus depletion of pools and accumulation of unrepaired DNA breaks may be responsible for cell death after UV.

The question whether the intrinsic activities of repair enzymes vary during the cell cycle is the subject of current investigations.

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CLONING OF A GENE THAT CONTROLS RADIATION SENSITIVITY, CELL DIVISION, AND CAPSULAR POLYSACCHARIDE SYNTHESIS, THE *lon(capR)* GENE[1]

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ABSTRACT Mutants in the *lon(capR)* gene of *E. coli* K12 are pleiotropic; i.e. 1) they form nonseptate filaments (*long* form) after UV or nitrofurantoin treatment; 2) in the absence of such treatments they overproduce capsular polysaccharide and synthesis of 10 enzymes involved in polysaccharide production is derepressed. Such clones appear mucoid. The *lon* gene product has been hypothesized to be a repressor. In order to identify and isolate this product, it appeared necessary to clone the *lon* gene. An F' episome containing the *lon*$^+$ and *proC*$^+$ genes was obtained by crossing an Hfr strain (with *proC*$^+$ as a late marker) with a *proC capR9 recA* strain and selecting for *proC*$^+$ and scoring for *lon*$^+$ (nonmucoid phenotype). One such clone contained an F' episome designated F'BZ105. The episome had a M.W. of 1.2×10^8 and could readily be isolated from CsCl-EtBr gradients. F'BZ105 DNA was mixed with pHA121 DNA (a plasmid that confers tetracycline resistance (Tc^R) and colicin E1 immunity and contains pSC101 and pHA105 (a 1.6 MD miniCol E1) joined at *EcoR*I sites), cut with *EcoR*I and ligated. A *lon(capR)* strain was transformed with the DNA and Tc^R clones were selected followed by replica plating onto minimal medium where nonmucoid (*lon*$^+$(*capR*$^+$)) clones could be distinguished. Three clones (of 6585 screened) yielded plasmids which could also transform *lon(capR)* strains to Tc, UV, and nitrofurantoin resistance and made them nonmucoid as well. UDP-glucose dehydrogenase, an enzyme that is elevated in the mucoid recipient, was repressed in the nonmucoid transformant. Two of these plasmids, designated pBZ201 and pBZ203, when cut with *EcoR*I each yielded a 6.1 MD fragment (pSC101) and a second fragment of 8.2 MD. pBZ201 and pBZ203 have been transformed into *lon* strains that also produce minicells in order to identify the polypeptides specified by the 8.2 MD fragment.

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INTRODUCTION

capR (a synonym for lon) mutants of E. coli K12 overproduce capsular polysaccharide (colanic acid) due to the derepression of the synthesis of 10 enzymes involved in polysaccharide production. Such mutants are also sensitive to ultraviolet and ionizing radiations and to treatment with nitrofurantoin in that nonseptate, inviable filamentous cells result from such exposures (1). The following work describes the cloning of a DNA restriction fragment from a capR$^+$proC$^+$ F-prime episome and the characterization of the cloned fragment with respect to capR$^+$ phenotype. The results substantiate the conclusion that the capR$^+$ gene has indeed been cloned.

RESULTS

When an E. coli K12 Hfr donor is crossed with a recA recipient and recombinants are selected for an allele near the origin or termination of transfer, the recombinants contain plasmids(2).Our initial step in the isolation of the lon(capR) gene was the in vivo generation of an F' episome of appropriate size as the result of a mating experiment with χ874 HfrOR54 (O purE....lac proC$^+$ capR$^+$ F) and the recipient JBC215 (F$^-$ proC capR9 recA). proC$^+$ exconjugants were selected, screened for the capR nonmucoid phenotype on minimal agar and the plasmid was further tested for the ability to confer resistance to UV radiation and nitrofurantoin (NF) by transfer to MC171 (a proC capR9 strain). F'BZ105, 1.2 x 10^8 daltons, was isolated from a conjugant recA clone as a plasmid DNA species by cleared lysate techniques in conjunction with CsCl-EtBr equilibrium density gradient centrifugation. Genes on such a plasmid are enriched 22.5-fold compared to the E. coli K12 genome which is 2.7 x 10^9 daltons.

A preparation of F'BZ105 plasmid DNA was used as the source of DNA fragments containing the capR$^+$ gene in a DNA cloning experiment. The plasmid utilized as the cloning vehicle, pHA121, is a composite plasmid of pSC101 and pHA105 (a 1.6 MD mini ColE1) joined at their EcoR1 sites, which confers both tetracycline resistance (Tc^R) and colicin E1 immunity (3). F'BZ105 and pHA121 DNA's were mixed, limit-digested with EcoR1 restriction endonuclease, and ligated with T4 ligase. This DNA preparation was used to transform lon(capR9) strains to Tc^R. Subsequent screening included replica-plating to minimal agar in order to distinguish the nonmucoid phenotype of lon$^+$(capR$^+$) clones. In one experiment 3 clones of 6585 yielded plasmid DNA which could transform several lon(capR) strains to Tc, UV, and NF resistance as well as the nonmucoid phenotype. When two of the plasmids, designated pBZ201 and pBZ203 were digested with EcoR1 each preparation

yielded a 6.1 MD fragment (pSC101) and a second fragment of 8.2 MD corresponding to a fragment observed in the EcoR1 fragment profile of F'BZ105. Other studies using restriction endonucleases HindIII, BamH1, and Sal1 indicated that the 8.2 MD fragments are arranged in opposite orientations in pBZ201 and pBZ203. Further characterization of bacterial strains containing pBZ201 or pBZ203 was pursued in order to demonstrate that capR$^+$ was indeed the gene cloned on the 8.2 MD DNA fragment. capR9 strains that were transformed with either pBZ201 or pBZ203 were nonmucoid (low colanic acid), contained repressed levels of UDP-glucose dehydrogenase (one of the enzymes that is repressed by the capR$^+$ gene (1)), and were UV and NF resistant.

The next phase of our study concerned the characterization of "variant" plasmid derivatives of pBZ201 and/or pBZ203. We observed segregation of Tc^R mucoid clones in many of the capR strains which had been transformed to the Tc^R nonmucoid phenotype by one of these plasmids. Also noted was a marked decrease in the frequency of such segregation of mucoid clones in capR9 recA strains transformed with capR$^+$ plasmids indicating that some of the segregants arose through recA-dependent recombination events between capR9 on the chromosome and capR$^+$ on the plasmid. Thus it appeared that the isolation of a capR9 plasmid from such mucoid segregants was feasible and might prove useful in the eventual identification of the capR$^+$ gene product.

Four separate plasmid DNA preparations were derived from independent mucoid segregants of several capR9 strains previously transformed to capR$^+$ by either pBZ201 or pBZ203. Early F'capR9/capR$^+$ partial diploid studies from this laboratory revealed a partial episomal dominance effect in that such bacterial strains were mucoid but UV^R (1). Two of the new plasmids transformed capR$^+$ strains to Tc^R and mucoidy; in contrast to F-prime partial diploids these transformants were UV^S and NF^S. We attribute these effects to the higher gene dosage and anticomplementation of capR9 subunits to inactivate capR$^+$ subunits with respect to UV resistance. Agarose gels revealed no marked differences in the restriction enzyme digest patterns of any of these preparations when compared with digests of pBZ201 or pBZ203, i.e. no large deletions or insertions occurred to produce these plasmids. These results, together with previous data (1, 4, 5) support the view that mucoidy and UV^S are both controlled by the product of a single cistron, the capR(lon) cistron.

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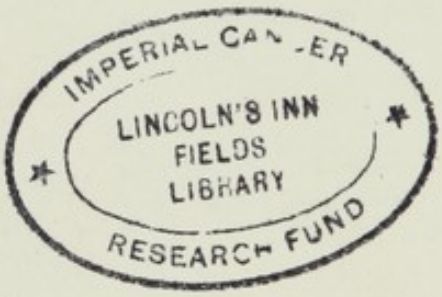
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