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CONTENTS:

Academic Press
NEW YORK SAN FRANCISCO LONDON
A Subsidiary of Harcourt Brace Jovanovich, Publishers
Academic Press, Inc.
111 FIFTH AVENUE, NEW YORK, NEW YORK 10003
Academic Press, Inc. (London) Ltd.
(Registered office registered number 598514)
24-28 OVAL ROAD, LONDON NW1 7DX
Harcourt Brace Jovanovich Group (Australia) Pty. Ltd.
P.O. BOX 300, NORTH RYDE, N.S.W. 2113, AUSTRALIA

ISBN 0–12–775168–8

MOLECULAR STRUCTURE OF HUMAN CHROMOSOMES

FROM THE PREFACE:

In recent years, a number of important developments have led to a dramatic increase in our understanding of chromosomes in biology and medicine. The sequence arrangement, transcriptional capacity, and functional significance of repeated and unique DNA sequences have been extensively studied. Refined investigations of chromosome and chromatin structure have been made possible with improvements in electron microscopic techniques, the use of premature chromosome condensation, and *in situ* hybridization. By the use of various dyes and treatments, mitotic chromosomes can now be visualized as having characteristic banding patterns, facilitating identification of individual chromosomes. Relationships between the human karyotype and those of other primates have been determined using differential staining techniques and comparative analysis of repeated DNA sequences. Gene mapping has been greatly accelerated with the use of rodent-human somatic hybrids.

Reviews of advances on the organization of the eukaryotic genome have been available for some time, yet they have not focused on the genome of greatest interest to us, that of Man. The aim of this volume is to fill this gap by bringing together authoritative contributions encompassing much of the knowledge available on the fine structure and molecular organization of the human genome.

Geneticists, molecular biologists, and cytogeneticists, particularly those interested in the human genome, will find this book to be an invaluable guide.

Jacket design by Jose R. Fonfrias

Molecular Structure of Human Chromosomes

CHROMOSOMES IN BIOLOGY AND MEDICINE

Edited by

JORGE J. YUNIS, M.D.

New Chromosomal Syndromes, 1977

Molecular Structure of Human Chromosomes, 1977

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ACADEMIC PRESS New York San Francisco London 1977

A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

Library of Congress Cataloging in Publication Data

Main entry under title:

Molecular structure of human chromosomes.

(Chromosomes in biology and medicine series)
Bibliography: p.
Includes index.
1. Human chromosomes. 2. Molecular genetics.
I. Yunis, Jorge J. II. Series: Chromosomes in biology and medicine. [DNLM: 1. Cytogenetics. 2. Chromosomes–Ultrastructure. 3. Molecular biology. QH600 M718]
QH600.M64 599'.9'048732 76-50672
ISBN 0–12–775168–8

PRINTED IN THE UNITED STATES OF AMERICA

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Preface

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Jorge J. Yunis

1

Molecular Organization and Function of the Human Genome

JORGE J. YUNIS, MICHAEL Y. TSAI,
and ANN M. WILLEY

I. INTRODUCTION

A major obstacle encountered in studying the genome of higher eukaryotes including man has centered around the large amount of DNA present in the nuclei of these cells. Compared to the extensively studied prokaryotic genome of *Escherichia coli*, consisting of 3.2×10^6 nucleotide pairs (Cairns, 1963), the genome of the lower eukaryote *Drosophila melanogaster* contains approximately

50 times that amount of DNA (Laird, 1971); while the human genome contains 1000 times the amount of DNA of *E. coli*, or 2.7×10^9 nucleotide pairs (Sober, 1970; Rees and Jones, 1972). Most of the prokaryotic genome is transcribed (McCarthy and Bolton, 1964; Kennell, 1968; Grouse *et al.*, 1972) and codes for approximately 3000 informational genes, while the *Drosophila* and human genomes have enough DNA to encode over 150,000 and 3 million such genes, respectively. Since man and other eukaryotes probably do not need such a large number of genes, explanations for the large amounts of DNA in eukaryotes have been sought.

II. RENATURATION STUDIES OF EUKARYOTIC DNA

The first step to the solution of the apparent enigma was the demonstration by Britten and co-workers of the presence of repetitive nucleotide sequences within the DNA of eukaryotic organisms (Bolton *et al.*, 1966; Waring and Britten, 1966; Britten and Kohne, 1968). Renaturation experiments done on a number of highly sheared animal DNA's have shown relatively constant proportions of repetitive and unique sequence DNA's (Britten and Kohne, 1968; Davidson *et al.*, 1974). In mammals, for example, repetitive sequences usually account for 30–40% of the genome, and unique sequences for the remaining 60–70%. Modeling after the prokaryotic system of gene regulation, transcription and translation, it was assumed that structural gene sequences would be represented in the unique copy DNA, whereas repetitive sequences were suspected of having regulatory functions (Britten and Davidson, 1969; Georgiev, 1969). Even under this assumption, however, the question remained as to the number of informational gene sequences present in the 60–70% of the genome consisting of unique copy DNA.

Detailed characterization of the arrangement of repetitive and nonrepetitive sequences was carried out in the genome of sea urchin and *Xenopus* (Davidson *et al.*, 1973; Graham *et al.*, 1974). The techniques employed included analyses of hydroxyapatite binding as a function of fragment length after low Cot incubation with carrier DNA's, measurement of repetitive sequence length by isolation of single-strand-specific, nuclease-resistant duplex on agarose gel column, and direct measurement with electron microscopy. The results of these studies revealed that unique copy DNA averaging 1000 nucleotides alternate with repetitive sequences averaging 200–400 nucleotides in about 50% of the genome. Two other patterns were also seen: long period interspersion of unique copy DNA several thousands of nucleotides in length with a few percent of repetitive sequences in about 40% of the genome, and clustered highly repetitive sequences in 5–10% of the total DNA. Similar patterns have since been reported in other eukaryotes (Firtel and Kindle, 1975; Angerer *et al.*, 1975). The only

organism which has been shown to have a large digression from the above pattern is *Drosophila melanogaster* (Manning *et al.*, 1975). In these animals, although the highly repetitive DNA sequences show a similar uninterrupted pattern, the remainder of the genome consists of interspersed unique and repetitive sequences covering a wide range with repetitive regions ranging from 500 to 13,000 base pairs averaging 5600, and unique copy stretches ranging from 2500 to 40,000 base pairs and averaging 13,000 nucleotides.

In man, Schmid and Deininger (1975) have recently reported a pattern of interspersed repetitive, single copy and inverted repeats in 50% of the genome. The average repetitive and nonrepetitive segments were thought to be 400 and 2000 nucleotides, respectively, although measurement by either electron microscopy or isolation of single-strand, nuclease-resistant duplexes was not carried out.

Evidence supporting the idea that unique copy sequences contiguous to repetitive DNA represent structural genes has been presented by Davidson *et al.* (1975) in the sea urchin genome. These authors have shown that 80–100% of the mRNA molecules present in sea urchin embryos are transcribed from single copy DNA sequences adjacent to interspersed repetitive sequences in the genome. However, single copy DNA finely interspersed with short repetitive sequences represents 40% of the total genome. As will be described in the following section, the implication that these large amounts of DNA all represent structural genes contradicts other estimates of gene number, which conclude only 1–6% of the eukaryotic genome represents informational genes.

III. GENE NUMBER AND GENOME TRANSCRIPTION

Several lines of evidence exist in favor of the tenet that only a small percentage of the eukaryotic genome represents structural genes. Ohta and Kimura (1971) postulated that less than 6% of the mammalian genome represents structural genes based on mutation rate and the resultant genetic load. In the giant salivary chromosomes of *Drosophila*, Judd and co-workers (Judd *et al.*, 1972; Judd and Young, 1974) observed that each chromomere represents one functional genetic unit, putting the total number of informational genes in this species at about 5000. In agreement with this view, it has been found that cytoplasmic polysomal RNA's from a large number of eukaryotes, including *Drosophila* and man, transcribe from approximately 2% of the genome (Greenberg and Perry, 1971; Galau *et al.*, 1974; Lewin, 1975). Moreover, Bishop *et al.* (1975) have shown that in *Drosophila* the total number of mRNA sequences expressed within the different stages of the life cycle of the fly do not exceed the 5000 chromomeres of the polytene chromosomes.

In contrast to this low amount of DNA responsible for mRNA transcription, it is known that a large amount of the DNA of the eukaryotic genome is transcribed but not translated. On the average, assuming asymmetrical transcription, total cellular RNA is transcribed from at least 10–30% of the single copy DNA of the total genome (Davidson and Hough, 1971; Gelderman *et al.*, 1971; Grouse *et al.*, 1972, 1973; Grady and Campbell, 1973; Turner and Laird, 1973). Additive experiments with RNA's from different organs showed that there is a considerable, although not total, overlap between RNA's from different tissues. In the case of the slime mold, it was shown that, overall, 56% of the genome is represented by transcripts between the amoeba and midculmination stages (Firtel, 1972).

A partial explanation for the discrepancy found between the large amount of transcribed DNA and the small percentage of the genome believed to represent messages can be found in recent studies on heterogeneous nuclear RNA (HnRNA). In mammalian cells, HnRNA was found to have more than five times the complexity of mRNA (Getz *et al.*, 1975), and in sea urchin embryos mRNA represents 2.7% of the genome, while 28.5% of the total single copy DNA hybridizes to HnRNA (Galau *et al.*, 1974; Hough *et al.*, 1975). Since there is also evidence that a large portion of the HnRNA of the sea urchin is composed of interspersed nonrepetitive and repetitive sequences (Smith *et al.*, 1974), it is possible that a large portion of the 50% finely interspersed single copy and repetitive DNA of the sea urchin is involved in HnRNA transcription.

HnRNA's are generally characterized by their overall rapid synthesis and degradation, large molecular weight, DNA-like base composition, their presence in all eukaryotes examined, and by the fact that the bulk of this class of molecules never leaves the nucleus (Sibatani *et al.*, 1962; Georgiev and Mantieva, 1962; Scherrer *et al.*, 1963; for review, also see Georgiev, 1974; Darnell, 1975). Besides the unique copy sequences of HnRNA that are known to have from 5 to 10 times the complexity of mRNA, HnRNA's also contain sequences transcribed from DNA of different degrees of repetitiveness. Holmes and Bonner (1974a) have suggested that in rat ascites cells, HnRNA molecules contain at least one middle repetitive sequence covalently attached to a single copy sequence. In these cells, HnRNA's are transcribed from approximately 12% of the genome, of which approximately 25% comes from repetitive and 75% from single copy DNA (Holmes and Bonner, 1974a,b). One type of repetitive sequence is characterized by its resistance to pancreatic RNase and is thought to be a double-stranded region that is formed by intramolecular base pairing. When denatured, the RNA sequences from the double-stranded regions, including those from HeLa cells, hybridize to DNA at a $Cot_{1/2}$ of about 10 (Jelinek and Darnell, 1972; Ryskov *et al.*, 1973a). A second type of repetitive sequence found in HnRNA of HeLa cells is largely (about 80%) made up of uridylic acid, is about 30 nucleotides in length and also hybridizes at a $Cot_{1/2}$ of about 10 (Molloy *et al.*, 1972). This oligo(U)

segment is located "only" in large (70–90 S) HnRNA molecules, is T_1 ribonuclease-resistant and pancreatic ribonuclease-sensitive. Recently, Molloy *et al.* (1974) found in HeLa cells that poly(A)-terminated HnRNA molecules longer than 20,000 nucleotides contain 2–3 units of oligo(U) nucleotides in fragments over 12,000 nucleotides away from the 3′ poly(A) ("messenger" end), while double-stranded regions are found between 3000 and 8000 nucleotides from this end.

That HnRNA may be the precursor of mRNA was suggested by a number of investigators soon after its discovery (Scherrer *et al.*, 1963; Penman *et al.*, 1963). Several models of genetic regulation have been proposed based on this concept (Scherrer and Marcaud, 1968; Georgiev, 1969, 1974; Darnell *et al.*, 1973). Although differing in many details, these authors all share the view that HnRNA's consist of a noninformative region, which presumably has some regulatory function, and an informative segment representing mRNA. In agreement with the view that messenger RNA's are formed from the posttranscriptional modification of higher molecular weight precursor RNA, it has been found that rRNA's are processed in a nonconservative fashion from 45 S RNA in which approximately 50% of the molecule is lost in the formation of 28 S and 18 S products (Maden, 1971; Choi and Saunders, 1974). Similarly, tRNA is processed from a higher molecular weight pre-tRNA (Burdon and Clason, 1969).

Several lines of experimental evidence exist implicating HnRNA as the precursor of mRNA (for review, see Darnell, 1975). For instance, sequences transcribed from integrated DNA of tumor virus and sequences present in hemoglobin and immunoglobulin have been found in HnRNA (Lindberg and Darnell, 1970; Melli and Pemberton, 1972; Imaizumi *et al.*, 1973; Williamson *et al.*, 1973). More recently, Herman *et al.* (1976) prepared cDNA to mRNA of HeLa cells and found that the bulk of the cDNA hybridized to isolated HeLa cell HnRNA. If HnRNA indeed contains mRNA as a part of its structure, the question then arises as to how it may participate in the regulation of mRNA formation. Although precise knowledge is not available, it is conceivable that the nonmessage part of HnRNA is needed either for transcriptional or posttranscriptional control of mRNA formation. In the former case, the nonmessage part may be transcribed from sites of recognition similar to those found in bacteria. The presence of possible recognition sites in eukaryotic cells has been proposed in several models to explain gene regulation in higher eukaryotes (Britten and Davidson, 1969; Georgiev, 1969, 1974; Darnell *et al.*, 1973). In contrast to the bacterial system, however, transcription of a large portion of nonmessage sequence into HnRNA raises the possibility of posttranscriptional regulation in addition to or in place of transcriptional control. Models for this type of regulation have been proposed (Scherrer and Marcaud, 1968; Darnell *et al.*, 1973) in which regulatory proteins would interact with HnRNA either to activate or suppress its processing into mRNA (Darnell, 1975).

IV. CORRELATION OF TRANSCRIPTION WITH SEQUENCE ARRANGEMENT PATTERNS

In correlating the information from recent studies of sequence arrangement and transcription of eukaryotes, many new questions arise. For instance, there is no satisfactory explanation for the discrepancy between the small amount of messenger RNA (1–2%) found and the large excess of repeat-contiguous single copy DNA (~40%) of putative structural genes. Even more puzzling is the function of long period interspersed single copy DNA which constitute an additional 40% of the total DNA and for which no specific function has been formulated.

Part of this problem stems from our lack of knowledge of the total complexities of messenger and heterogeneous nuclear RNA that are expressed in different stages of development and in different cell types. Additive studies such as those performed in *Drosophila* (Bishop *et al.*, 1975) on the complexities of mRNA should be extended to mammalian HnRNA and mRNA as well to obtain independent determination on the approximate number of structural genes in different species. On the other end of the spectrum are the difficulties encountered in interpreting the results obtained from sequence arrangement studies. Since DNA renaturation techniques are used in these studies, the results heavily depend on the fidelity of duplex reassociation. This process is best described as an "association" because, when dealing with repetitive sequences, the two strands forming the duplex may not be identical to the original paired strands, and mismatching due to base substitution would greatly affect the structures seen. Therefore, it is difficult to be certain that all of the nonassociated sequences adjacent to repetitive segments are representative of unique copy DNA and not of repetitive components with large accumulations of base substitutions. It is conceivable that, just as intermediate repetitive sequences may have evolved from simple sequences disguised by mismatching, further base substitution can cause such sequences to reassociate as single copy DNA (Walker, 1971).

V. FRACTIONATION OF NATIVE DOUBLE-STRANDED DNA

A different approach in the study of the eukaryotic genome makes use of fractionation of total native DNA through the use of cesium salt density gradient fractionation. Progress was first made with this technique in the early 1960's when minor components differing in buoyant densities from the bulk of the DNA were observed in the mouse, guinea pig, calf, and crab when centrifuged in neutral cesium chloride density gradients (Kit, 1961,

1962; Szybalski, 1961; Walker and McClaren, 1965). These minor components were subsequently termed satellite DNA which are characterized as being composed of short sequences from 6 to 30 nucleotide pairs repeated as many as several million times (in mammals, Cot $\sim 10^{-5}$–10^{-2}) in long uninterrupted blocks of DNA largely localized in pericentromeric and perinucleolar heterochromatin (for review, see Yunis and Yasmineh, 1971). In man, four relatively well-characterized satellite DNA's have been isolated, mainly with the aid of the technique of metal-cesium sulfate density gradient centrifugation (Corneo *et al.*, 1967, 1970, 1971, 1972; Corneo, 1968; see also Chapters 2 and 3). Because of the short repeat size, unusual base sequence, accumulation of base substitutions (Southern, 1970, 1972; Salser *et al.*, 1976), species differences in satellites (Flamm *et al.*, 1969; Walker *et al.*, 1969; Hennig and Walker, 1970; Yunis and Yasmineh, 1971), and the lack of cellular RNA's which hybridize to satellite DNA (Flamm *et al.*, 1969; Melli *et al.*, 1975), it seems unlikely that these sequences code for proteins. It has therefore been suggested that this class of DNA would be involved in maintaining the centromeric structure and function, protecting other vital regions of the genome such as the 18 S and 28 S ribosomal cistrons and serving as an effective barrier against cross fertilization among related species (Yunis and Yasmineh, 1971; Sutton, 1972).

Techniques of fractionating native DNA received renewed interest when Filipski *et al.* (1973) showed that calf DNA can be separated into three components that band at discrete densities in CsCl (1.697, 1.704, and 1.709 gm/cm^3) and represent 50, 25, and 10% of the DNA, respectively. The three major components observed in the calf have been seen, by the same procedure, in high molecular weight DNA's of three more mammals (man, guinea pig, and mouse). The 1.704 and 1.709 gm/cm^3 components (corresponding to 25 and 10% of the genome, respectively) did not show any variation in density and only very slight differences in relative amounts in other mammalian DNA's. In contrast, the main component (1.697–1.699 gm/cm^3) was found to subdivide into two subcomponents and showed species differences (Thiery *et al.*, 1976; see also Chapter 2).

Recently, Yunis *et al.* have reported a CsCl density gradient hybridization technique allowing the identification of biased density native DNA components containing repetitive sequences (Yasmineh and Yunis, 1974, 1975; Willey and Yunis, 1975, 1977a,b; Sanchez and Yunis, 1976; Tsai and Yunis, 1977). This method generally involves the hybridization of radioactively labeled RNA transcribed from repeated sequences to single-stranded ends of native nuclear DNA, and determination of buoyant densities of the hybrids in neutral CsCl gradients (Fig. 1). Using the hybrid peaks as markers, the genome can then be fractionated into several native components from preparative cesium salt gradients. The combined use of CsCl density gradient hybridization and relaxed Cs_2SO_4/Ag^+ density gradient centrifugation has facilitated the preliminary fractionation of

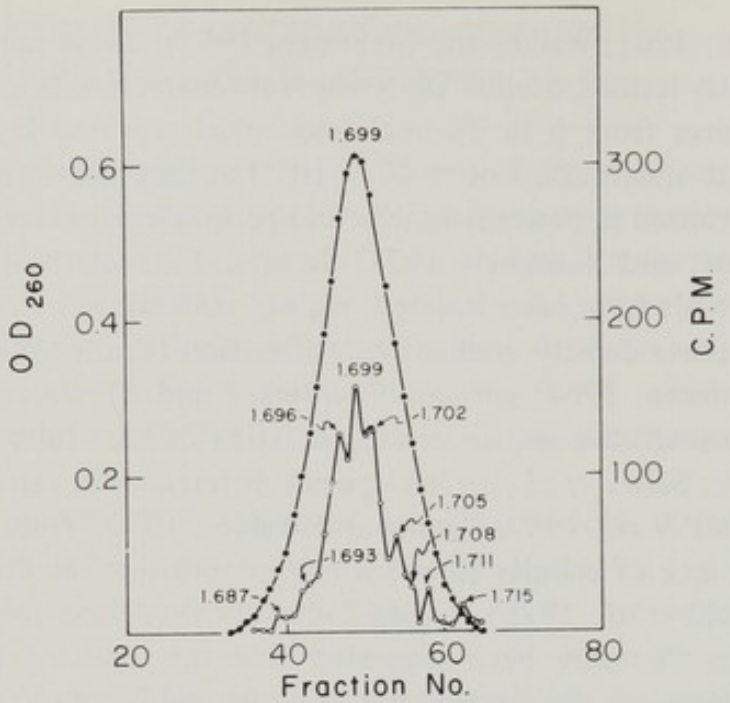

Fig. 1. Neutral CsCl density gradient centrifugation of human RNA/DNA hybrids. Complementary RNA (cRNA) was transcribed from total repetitive DNA and hybridized to single-stranded ends of DNA generated by mild shearing through a 25 gauge needle ten times at room temperature. Incubation was carried out for 16 hours at 65°C in 2 × SSC. RNA that failed to hybridize was separated from DNA through an agarose column (BioGel A-5M). Each 13 ml gradient was fractionated with an ISCO density gradient fractionator. DNA was read with a UV monitor at 260 nm and 0.08 ml fractions were collected in scintillation vials. The numbers (with arrows) in the figure represent buoyant densities of the DNA at the peaks of radioactivity. Abscissa, fraction number; left ordinate, OD_{260}; right ordinate, cpm of each fraction counted in a mixture of 10 ml Aquasol and 1 ml H_2O. DNA, •——•; cRNA, ○——○. (From Tsai and Yunis, 1977.)

human DNA into seven major components that band at 1.698, 1.700, 1.702, 1.705, 1.708, 1.711, 1.715 gm/cm^3, respectively, in neutral cesium chloride density gradients (Sanchez and Yunis, 1976) (Fig. 2). The components that band at 1.705 and 1.708 gm/cm^3 were found to comprise approximately 25 and 10% of the total DNA, respectively, as observed in other mammals (Chapter 2).

In the Algerian hedgehog, using cRNA prepared from total repetitive DNA (Cot < 100) for the CsCl gradient hybridization technique, three major hybrid peaks sedimenting at densities of 1.702, 1.705, and 1.708 gm/cm^3 were observed. Recycling in neutral CsCl gradients showed that it was possible to isolate native DNA components at these densities, and that these components amounted to approximately half of the genome (Willey and Yunis, 1977b) (Fig. 3). To learn about the possible nature of the kind of repetitive DNA present in each component, cRNA to various Cot fractions was obtained and hybridized to total nuclear DNA. Highly repetitive DNA (Cot $< 10^{-3}$; 14% of the genome) showed an enrichment in the 1.702 gm/cm^3 hybrid peak; moderately repetitive

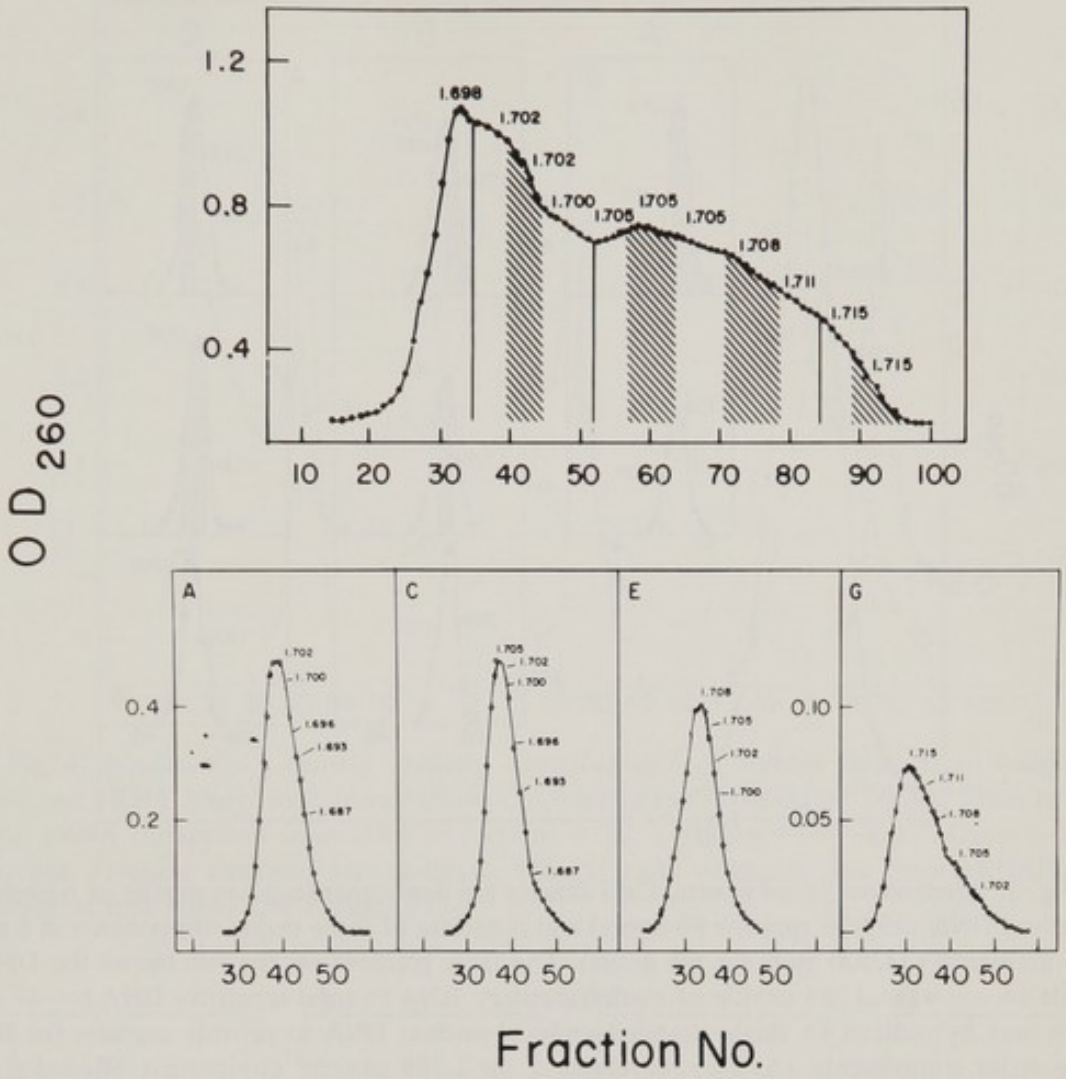

Fig. 2. Top: relaxed $Cs_2SO_4 - Ag^+$ density gradient centrifugation of total nuclear human DNA of molecular weight 3.6×10^6 daltons. Centrifugation was carried out for 90 hours at 39,000 rpm followed by 20 hours of reduced speed at 26,000 rpm. The Ag^+/DNA-P ratio was 0.32. Eleven pools were made of the fractions, and the buoyant densities of these pooled DNA in neutral CsCl density gradient centrifugation are indicated on top of the fractions pooled. Bottom: the neutral CsCl density gradient profiles of four of these pooled fractions (shaded areas in the top figure) are illustrated. (From Sanchez and Yunis, 1976.)

DNA (Cot $< 10^{-3}$ to 1; 23% of the genome) showed enrichment in the 1.708 gm/cm^3 hybrid peak; a slightly repetitive DNA (Cot 1–100; 14% of the genome) showed enrichment in the 1.705 gm/cm^3 hybrid peak (Willey and Yunis, 1975) (Fig. 4).

Using a similar approach in *Microtus agrestis*, a major native DNA component sedimenting at 1.703 gm/cm^3 was observed. This component was found to be rich in moderately repetitive DNA (Cot 10^{-2}–5), amounting to approximately 17% of the genome. It is partly interspersed with two satellite DNA's (8% of the genome) and preferentially localized in the giant heterochromatic sex chromo-

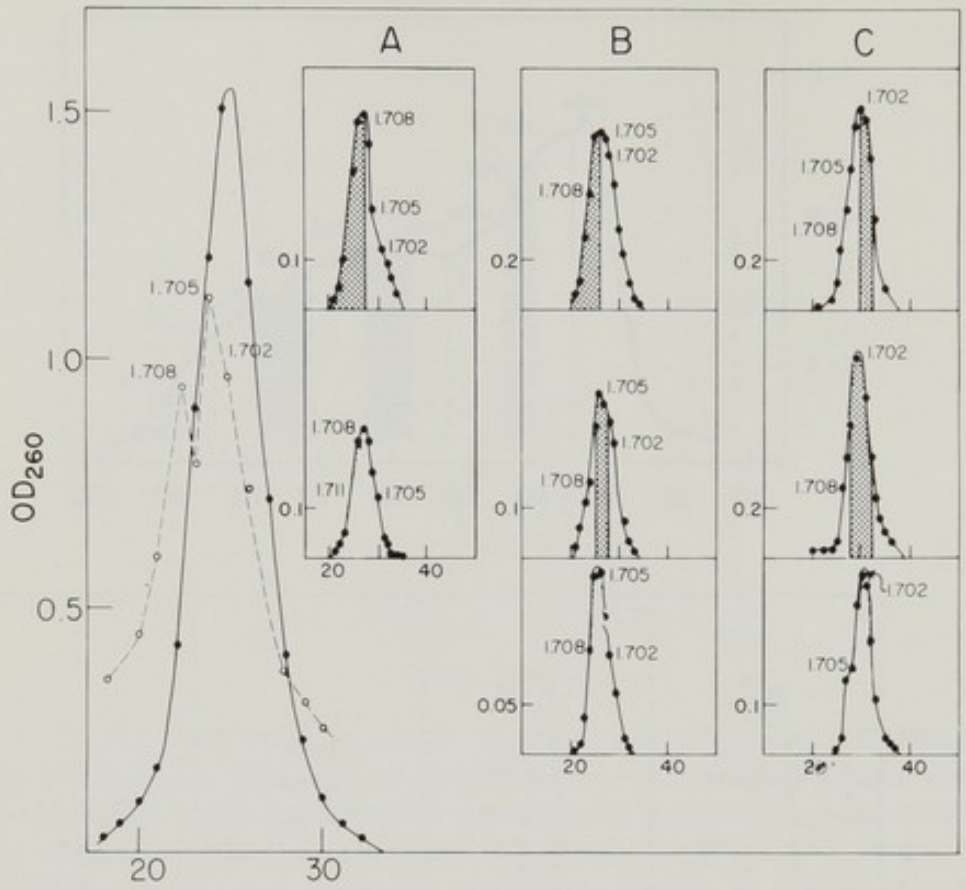

Fig. 3. Preparative 30 ml neutral CsCl density gradient centrifugation profile of Algerian hedgehog DNA (29,000 rpm for 90 hours) and recycling of three major components in 6 ml CsCl gradients (33,000 rpm for 60 hours). The large gradient on the left shows the DNA profile (●——●) and the profile of complementary RNA to total repetitive DNA (○——○) which was hybridized to single-stranded ends of nuclear DNA to provide markers for the three major components. (A) Two recyclings of the 1.708 gm/cm^3 component. Shaded area of upper gradient was recycled in the lower gradient. (B) Three recyclings of the 1.705 gm/cm^3 component. Shaded area indicates the fractions which were recycled. (C) Three recyclings of the 1.702 gm/cm^3 component. (From A. M. Willey and J. J. Yunis, unpublished.)

somes of this species which, in turn, contain approximately 20% of the total DNA (Yasmineh and Yunis, 1971, 1973, 1975) (Table I; Figs. 5 and 6). The finding that the DNA of the giant chromosomes of *Microtus agrestis* is composed primarily of moderately repetitive DNA (Cot 10^{-2}–5) extends that portion of the genome of eukaryotes that resides in heterochromatin beyond the highly repetitive satellites DNA's, and correspondingly decreases the amount and type of repeated sequences that may be finely interspersed with structural genes in the total genome.

In man, components with interspersed moderately and highly repetitive sequences have also been reported (Marx *et al.*, 1976). These authors found that DNA sheared and freed from foldback sequences sediment as five distinct families in cesium chloride density gradients when reassociated to a Cot of 1.0.

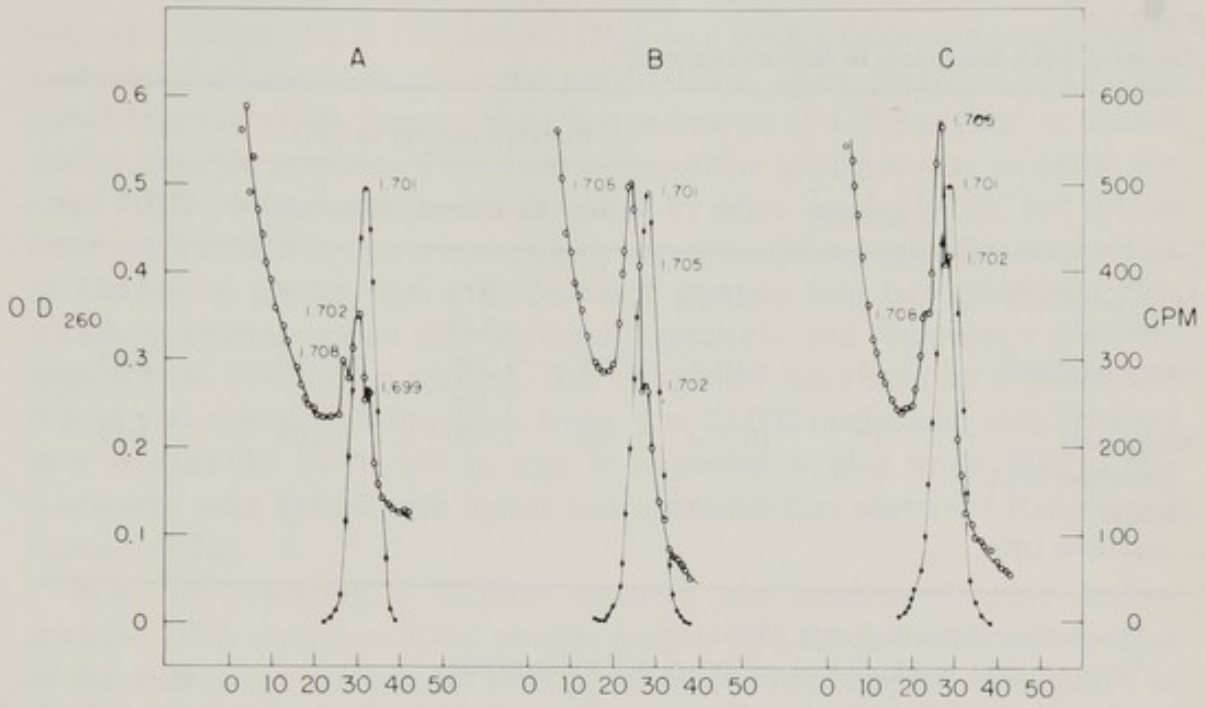

Fig. 4. Neutral CsCl density gradient centrifugation of hybrid forms from hedgehog DNA and cRNA transcribed from various Cot fractions of reassociated DNA's. From left to right, cRNA transcribed from DNA of (A) Cot $< 10^{-3}$; (B) Cot = 10^{-3}–1; (C) Cot = 1–100. Abscissa, fraction number; left ordinate, OD_{260}; right ordinate, cpm from cRNA/DNA hybrids. (From Willey and Yunis, 1975.)

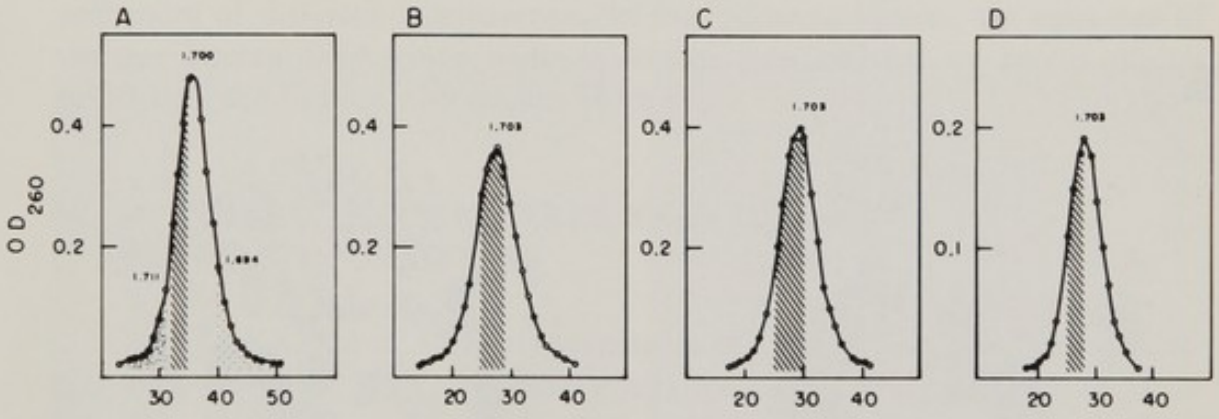

Fig. 5. Neutral CsCl density gradient centrifugation of *Microtus agrestis* DNA and recycling of the moderately repetitive component (Cot = 10^{-2}–5). (A) Preparative gradient loaded with 28 OD_{260} units of DNA from liver heterochromatin (MW 1.7×10^6 daltons) in a final volume of 28 ml. Following centrifugation at 30,000 rpm for 90 hours, 0.5 ml fractions were collected from the bottom of the gradient. The speckled and dotted areas represent a heavy and a light satellite DNA, respectively, whereas the dashed area was recycled in neutral CsCl density gradients to band at 1.703 g/cm^3. (B–D) Further recyclings of the dashed area in (A) were carried out and the dashed area in (D) used as template for cRNA synthesis. See Fig. 6. (From Yasmineh and Yunis, 1975.)

TABLE I
Repeated DNA Sequences in *Microtus agrestis*[a]

	% genome	Cot	Buoyant density in CsCl			AT/GT ratio
			Native	Denatured	Reassociated	
Light satellite[b]	3	$<10^{-3}$	1.694	1.770 1.755	1.770	2.00
Heavy satellite[b]	5	$<10^{-3}$	1.711	1.769 1.756	1.716	1.03
Moderately repetitive DNA[b]	17	10^{-2}–5	1.703	1.760	1.708	1.54
Slightly repetitive DNA	~6	5–100	–	–	1.715	–

[a] From Yasmineh and Yunis, 1975.
[b] Preferentially localized in constitutive heterochromatin.

Of the five, three possess satellite-like properties (6% of the genome). The remaining two band at 1.703 and 1.714 gm/cm^3 (7 and 9% of total DNA, respectively), and each consists of one-fourth highly repetitive and three-fourths intermediate repetitive DNA that renature with $Cot_{1/2}$ of 14 and 7.2, respectively. A clustered intermediate repetitive sequence component has also been

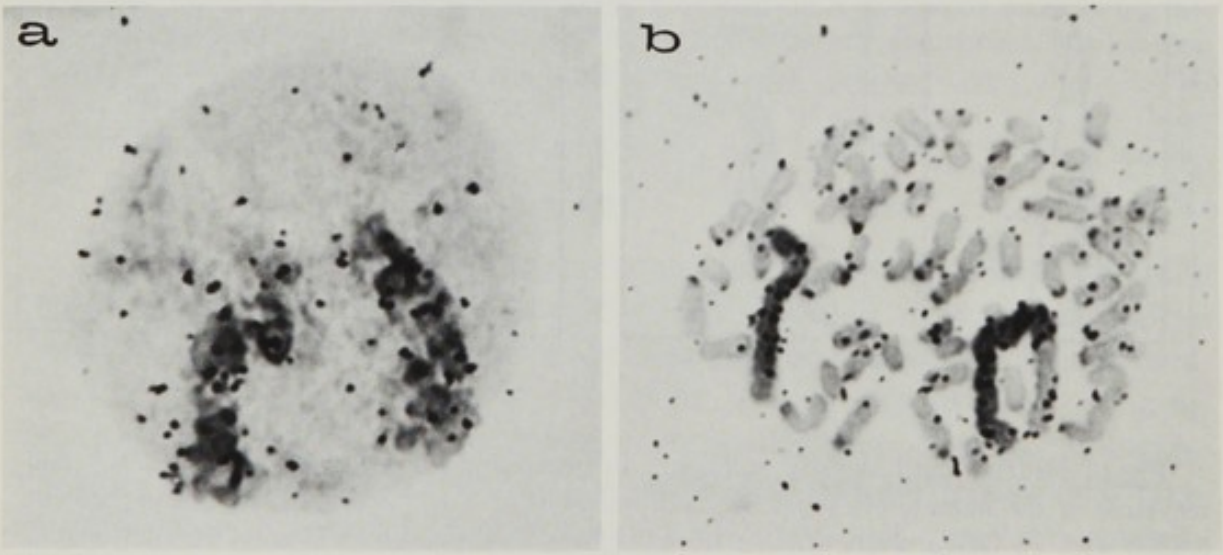

Fig. 6. Autoradiograph of (a) an interphase nucleus and (b) a metaphase of *Microtus agrestis* bone marrow hybridized with tritiated RNA complementary to moderately repetitive DNA (Fig. 5D). Note the preferential labeling over the large segments of constitutive heterochromatin of the giant sex chromosomes. Stained with Giemsa. (From Yasmineh and Yunis, 1975.)

reported in man by Corneo *et al.* (1975). These authors fractionated large molecular weight (1.5×10^7 daltons) DNA on a MAK column and found that in a few fractions the large molecular weight DNA, when denatured and reassociated to a Cot of 20, forms a band that sediments at 1.703 gm/cm^3 in neutral cesium chloride gradients. This clustered repetitive sequence may represent the same 1.703 gm/cm^3 component described by Marx *et al.* (1976), and differs from satellite DNA in that it is not highly repetitive (found only after reannealing at Cot values greater than 10), does not separate into two complementary strands in alkaline cesium chloride density gradients, and comprises a relatively large portion (7%) of the genome. This component has a complex organization; it is in part clustered in fragments larger than 24,000 nucleotide pairs, behaving as a satellite-like DNA, and in part in fragments ranging from 1800 to 600 nucleotide pairs spaced with longer and more complex sequences (Ginelli and Corneo, 1976).

From the foregoing, it becomes apparent that isolation of native double-stranded DNA components can provide insights into the molecular organization of the mammalian genome not revealed by renaturation studies. As suggested in the case of the Algerian hedgehog genome, major isolated DNA components may have a more homogeneous content of repetitive sequences compared to total DNA. In addition, blocks of moderately repetitive sequences (Cot $\sim 10^{-2}$–1), interspersed with highly repetitive DNA (Cot $< 10^{-3}$), were found in the genomes of *Microtus agrestis* and man. These results are at variance with those obtained from renaturation studies, which did not generally uncover blocks of sequences of different repetitiveness. In the following section, the existence of discrete human DNA components is further substantiated by recent studies involving hybridization with nuclear RNA's.

VI. HYBRIDIZATION OF NUCLEAR RNA's TO DNA IN CsCl DENSITY GRADIENTS

Nuclear RNA's transcribed from repetitive DNA sequences have been isolated and hybridized to DNA in CsCl density gradients. The results from these experiments provide further suggestive evidence that human DNA in CsCl density gradients is made up of several components having discrete biased densities. For instance, Melli *et al.* (1975) hybridized radiolabeled HnRNA to CsCl density gradient fractions of high molecular weight repeated sequences of HeLa DNA. Instead of finding an even distribution of the sequences over main band DNA, as might have been expected for fine interspersion, the results showed at least three radioactive peaks of biased densities. The predominant peak lies on the heavy side of main band DNA whereas two smaller peaks fall to the light side (Fig. 7).

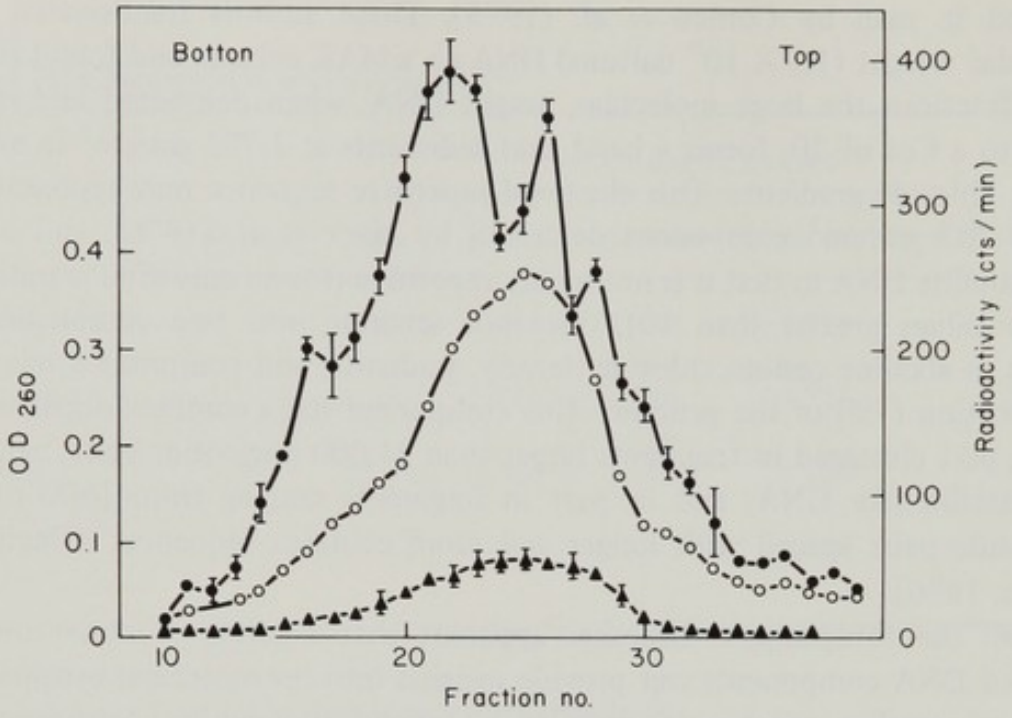

Fig. 7. Hybridization of HeLa HnRNA to fractions of DNA from a cesium chloride density gradient. Placenta DNA, 140 μg (MW 6 × 10^7 daltons), was centrifuged to equilibrium in a cesium chloride gradient. DNA fractions (○——○) were collected from the bottom, denatured in 0.1 *M* NaOH, and diluted with 2 *M* NaCl to pH 11.4. Samples from each fraction were loaded onto Millipore filters and hybridized for 3 hours with ^{3}H-labeled HnRNA (5 × 10^4 cpm/ml) before and after heat treatment, respectively. The RNase-resistant hybrids were measured in duplicates. The profile is drawn through the average of the two values for the boiled (●——●) and untreated (▲——▲) RNA. The extreme values of the vertical bar lines mark the two estimates where these are significantly different. (From Melli *et al.*, 1975).

More recently, Tsai and Yunis (1977) investigated the hybridization pattern of chromosomal RNA to human DNA. Chromosomal RNA is a small molecular weight nuclear RNA characterized by a high content of dihydropyrimidine (8–11%). Over 80% of this class of molecule is thought to be transcribed from intermediate repetitive DNA ($Cot_{½}$ = 1.0) (Holmes *et al.*, 1974). As shown in Fig. 8, hybridization of ^{125}I-labeled human placental chromosomal RNA to total DNA by the CsCl density gradient hybridization technique revealed three radioactive peaks corresponding to DNA with densities of 1.702, 1.705, and 1.708 gm/cm^3 (Tsai and Yunis, 1977). The fact that this small molecular weight nuclear RNA, like HnRNA, may not be evenly dispersed throughout the bulk of the human DNA is of interest. Taken together, the pattern of hybridization of HnRNA and chromosomal RNA's to DNA in cesium chloride density gradients provides suggestive evidence that the components of DNA that band at discrete densities may have different functional roles.

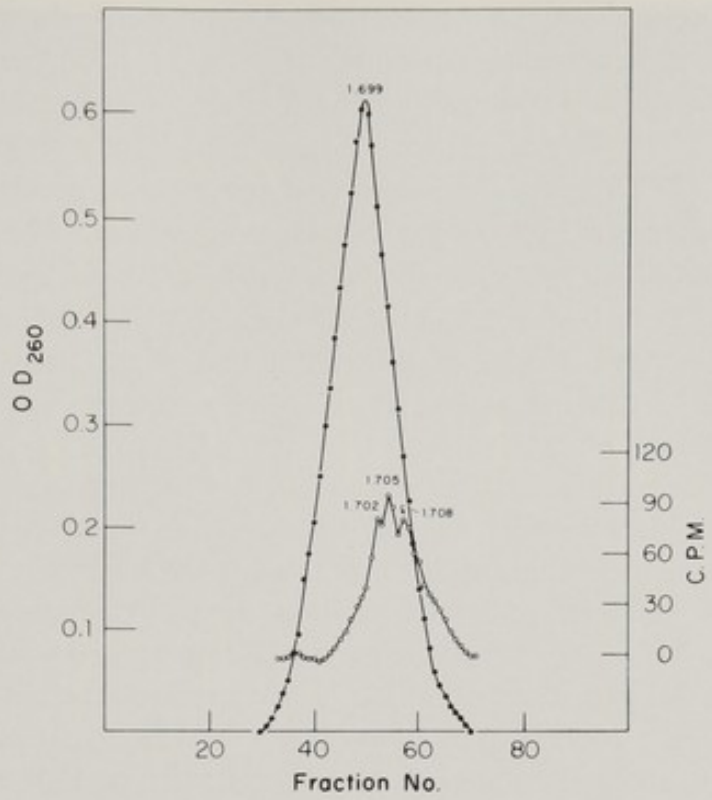

Fig. 8. Neutral CsCl density gradient centrifugation of human placenta ^{125}I-labeled chromosomal RNA hybridize to single-stranded ends of human DNA (MW 4.5 × 10^6 daltons). Hybridization was carried out at 65°C for 16 hours in 2 × SSC. Details of the procedures are described in Fig. 1. Abscissa, fraction number; left ordinate, OD_{260}; right ordinate, cpm of each ml fraction counted in a mixture of 10 ml Aquasol and 1 ml H_2O. DNA, •——•; chromosomal RNA, ○——○. (From Tsai and Yunis, 1977.)

VII. CHROMOSOMAL LOCALIZATION OF SPECIFIC DNA AND RNA COMPONENTS

Study of the molecular organization of the genome can be extended to the chromosomal level by *in situ* hybridization. As described below, this technique has been applied for the localization of highly and intermediate repetitive DNA's as well as RNA transcripts of intermediate repetitiveness, such as those of chromosomal RNA and low multiplicity gene copies. Satellite DNA sequences have been found preferentially localized in regions of constitutive heterochromatin, particularly around centromeres and nucleolar organizers (for review, see Yunis and Yasmineh, 1971; Jones, 1973). In man, as in other higher eukaryotes, the highly repetitive satellite DNA sequences generally account for approximately 6–10% of the genome (Corneo *et al.*, 1973; Jones, 1973; Saunders *et al.*, 1975). Satellite DNA I sediments at 1.687 gm/cm^3 in CsCl density gradients, is

preferentially localized to the pericentromeric region of chromosome 9, the short arm of the acrocentric chromosomes (13–15, 21–22) (Jones *et al.*, 1975) and the distal half of the long arm of the Y chromosome (Evans *et al.*, 1974; Gosden *et al.*, 1975). Satellite II sediments at 1.693 gm/cm^3 and is found on chromosomes 1, 9, 16 and the acrocentric chromosomes, particularly on chromosome 21 (Gosden *et al.*, 1975). Satellite III sediments at 1.696 gm/cm^3 and is found on chromosome 9, and the acrocentric chromosomes, particularly in chromosome 21 (Gosden *et al.*, 1975). Satellite IV sediments at 1.700 gm/cm^3 and has major blocks on the distal half of the long arm of the Y chromosome and on pericentromeric regions of chromosomes 13, 14, 15, 21, 22, and 20 (Gosden *et al.*, 1975).

The localization of blocks of satellite DNA's in the heterochromatic centromeric regions of chromosomes and the known inference with crossing-over by constitutive heterochromatin suggests that satellite DNA may lead to the relative conservation of genes immediately adjacent to it. A tendency to suppress crossing-over, to influence linkage conservation and to predispose to chromosome fusion on the part of satellite-rich heterochromatin provides an adequate explanation for its structural role (Chapter 9). The fact that different species possess different satellite components also suggests that satellite DNA evolves in step with speciation and serves as a barrier against cross fertilization (Yunis and Yasmineh, 1971).

In situ localization of intermediate repetitive sequences has also been examined in several organisms. In *Drosophila*, for example, the majority of the intermediate repeated sequences are localized on β-heterochromatin and a small percentage is widely distributed among the chromomeres of all chromosomes with preference to the X (Gall *et al.*, 1971); in *Microtus agrestis*, a component sedimenting at 1.703 gm/cm^3, rich in moderately repetitive DNA (Cot 10^{-2}–5), and amounting to 17% of the genome, was found partly interspersed with two satellites (8% of the genome) and preferentially, but not exclusively, localized in the giant heterochromatic sex chromosomes of this species (20% of the chromatin) (Yasmineh and Yunis, 1971, 1973, 1975) (Fig. 6). The interspersion of moderately and highly repetitive sequences within blocks of heterochromatin suggests that these moderately repetitive sequences may have a similar function. Also, in the Algerian hedgehog, moderately repetitive DNA (Cot 10^{-2}–1), corresponding to approximately 23% of the genome, appears to be largely present in a major native DNA component sedimenting at 1.708 gm/cm^3 and is preferentially localized to intercalary heterochromatin and Q-positive bands (Willey and Yunis, 1975, 1977b).

In man, moderately repetitive DNA sequences are preferentially localized in Q-positive bands (Sanchez and Yunis, 1974) (Fig. 9). Human chromosomes have no known intercalary heterochromatin, but some chromosomes, particularly 13 and 18, possess large G-positive bands and a disproportionately large amount of repetitive DNA. These regions are of medical importance because of the fact that

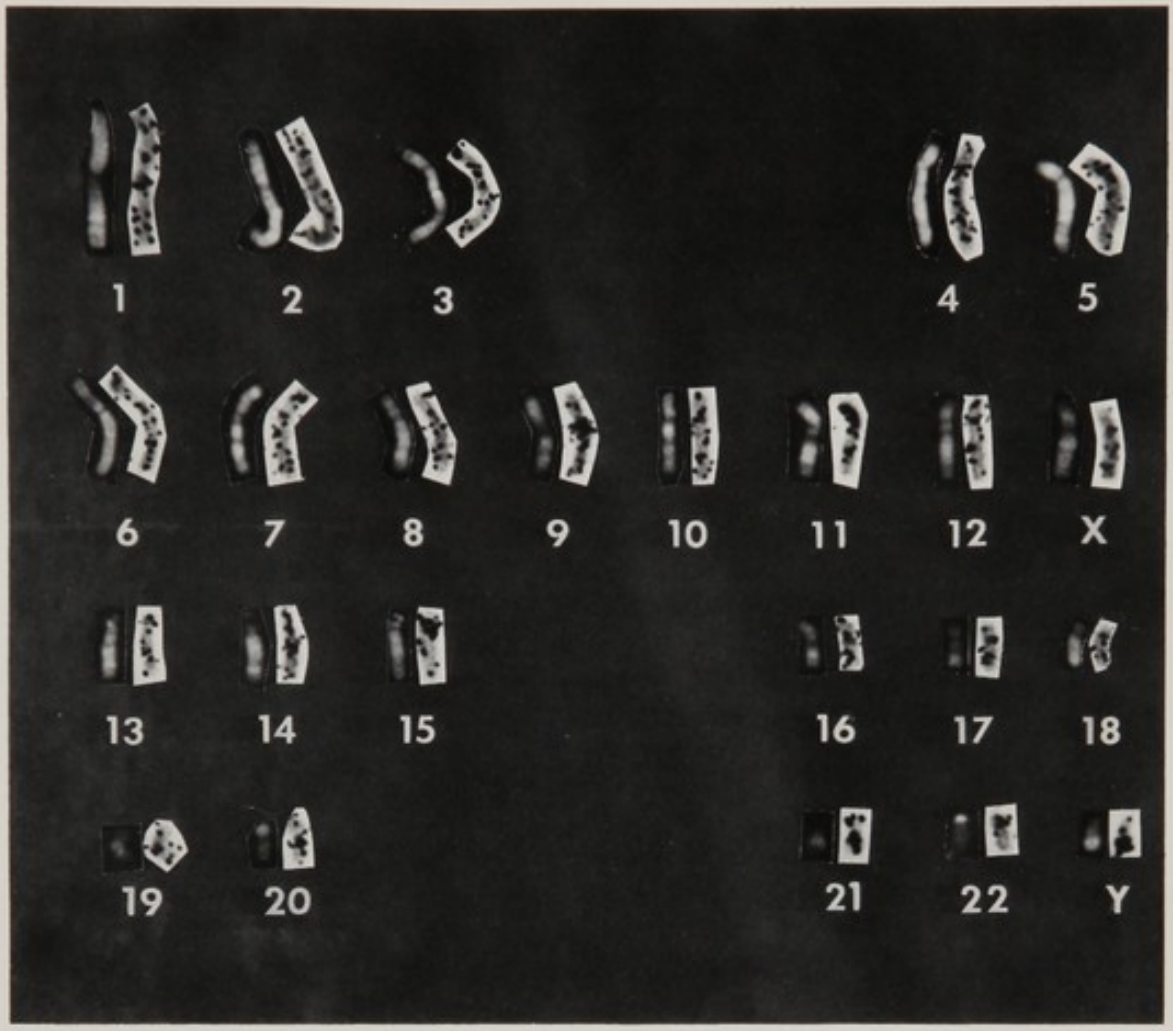

Fig. 9. Haploid set of a human metaphase stained with quinacrine mustard and subsequently exposed to cRNA to moderately repetitive DNA. In each pair the Q-banded chromosome is on the left and the cRNA labeled one is on the right. Note the close correspondence between Q-positive bands and labeling patterns. The pericentromeric regions and some telomeres are also labeled. (From Yunis, 1974.)

most of the well-documented large autosomal defects of man have been found to involve chromosomes with large amounts of G-positive, late replicating, and presumably heterochromatic-like material (Yunis, 1965, 1974).

A special class of repeated sequences, called inverted repetitive sequences, hairpin or foldback DNA, has recently been characterized in eukaryotes. In man, these sequences comprise 3–6% of the total DNA (Wilson and Thomas, 1974; Schmid and Deininger, 1975; Dott *et al.*, 1976), have a length range of 300–1200 nucleotides and reassociate with unimolecular kinetics ($Cot < 10^{-5}$). Single-stranded, nuclease-resistant regions of this class of DNA contain sequences representative of almost all repetition frequencies, including very highly repetitive as well as very few copy sequences. They have been recently found to be widely distributed throughout metaphase chromosomes (Dott *et al.*, 1976). The

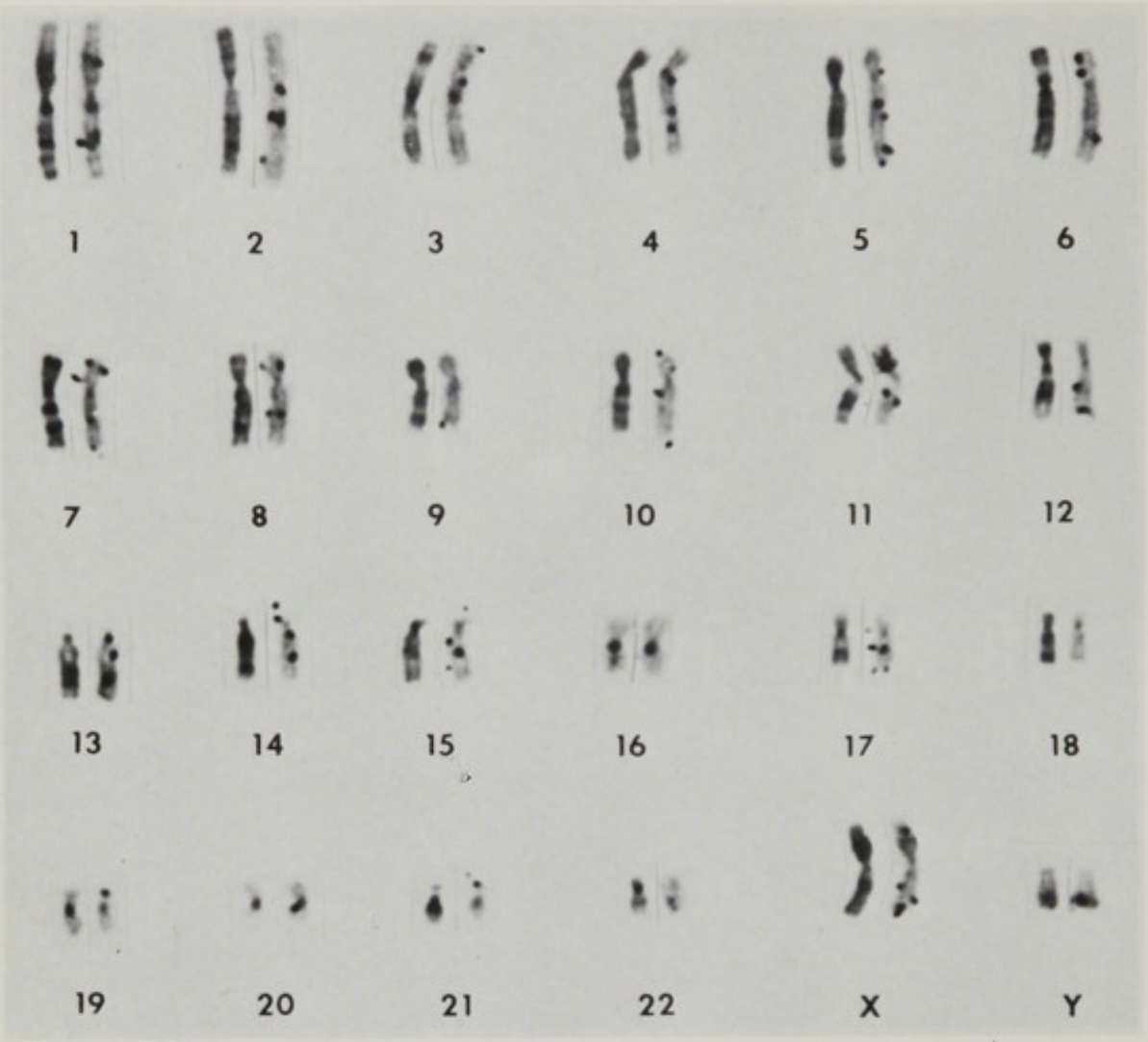

Fig. 10. Haploid set of human metaphase chromosomes stained with Giemsa and subsequently hybridized to ^{125}I chromosomal RNA obtained from human placenta. In each pair the G-banded chromosome is on the left and the labeled chromosomal RNA is on the right. Note the close correspondence between G-positive bands and labeling patterns. Some of the telomeres and the short arm of acrocentric chromosomes are also labeled. (From Pierpoint and Yunis, 1977.)

function of foldbacks is not clear. Recently, Ryskov *et al.* (1973b) and Church *et al.* (1974) have presented evidence suggesting that foldbacks may, in part, represent sequences of HnRNA that form secondary structures.

In situ hybridization with transcripts of intermediate repetitive sequences has recently been carried out (Pierpont and Yunis, 1977). Using ^{125}I-labeled human chromosomal RNA, it was found that this small molecular weight nuclear RNA has a preferential localization to the telomeric regions and heterochromatic short arm of acrocentric chromosomes as well as significant hybridization to Q/G-positive bands (Fig. 10).

Transcripts of tandem gene duplicates are being extensively studied. Of these, ribosomal cistrons have been localized by *in situ* hybridization in man. Accord-

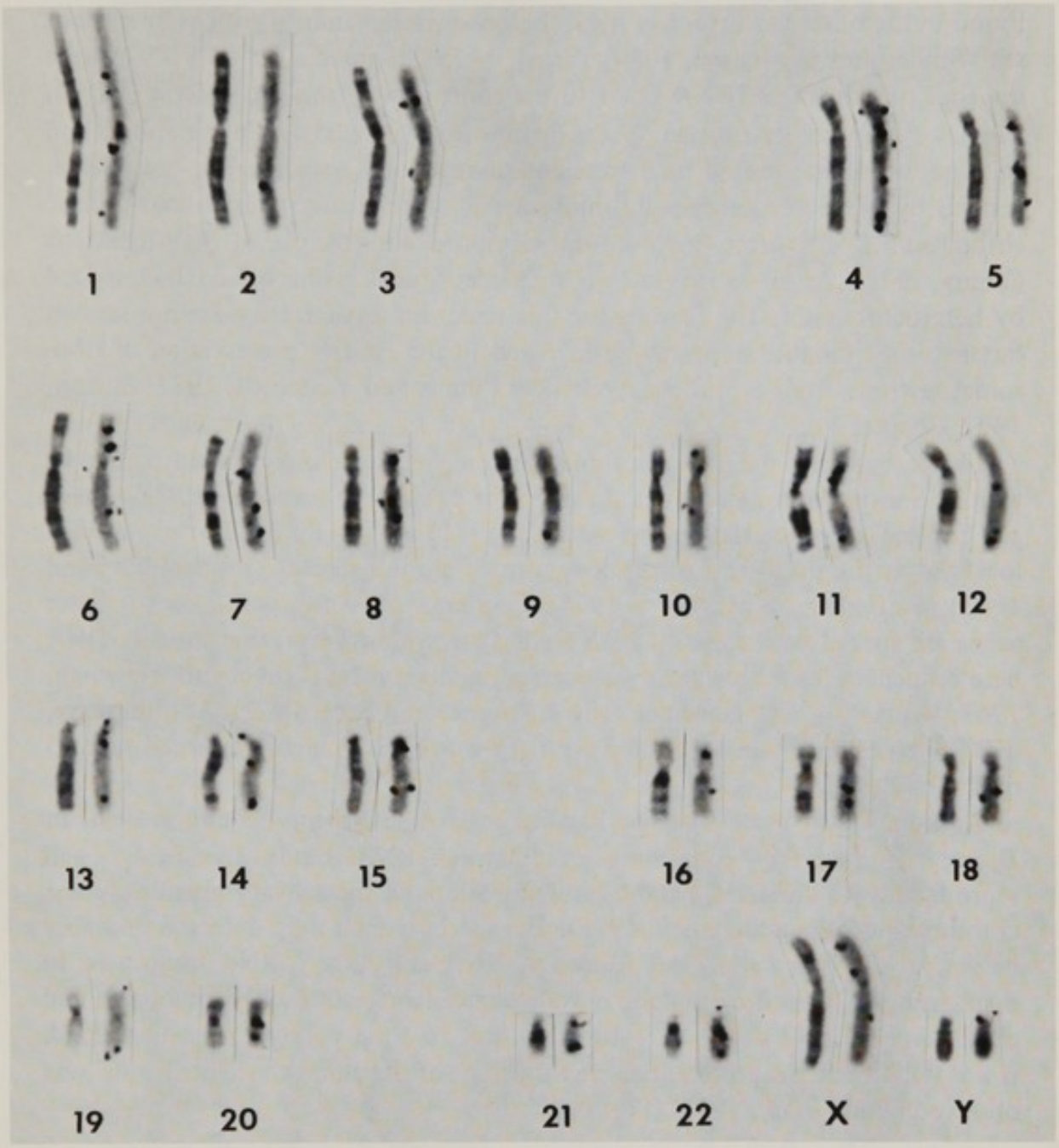

Fig. 11. Haploid set of a human male metaphase stained with Giemsa and subsequently hybridized to cDNA from total poly(A) cytoplasmic RNA. For each chromosome, the G-banded chromosome is on the left and the same chromosome after *in situ* hybridization with labeled cDNA is on the right. Note a general but not exclusive correspondence between G-negative bands and labeling patterns. (Yunis *et al.*, 1977.)

ing to Bross and Krone (1972), the human genome has 213 nucleolar rDNA cistrons per haploid set and the genes that code for ribosomal RNA's are always found in the nucleolar organizer in all the organisms examined so far (for review, see Wimber and Steffensen, 1974; Tartof, 1975). Henderson *et al.* (1972) were the first to map these rDNA genes to the short arm of the acrocentric (13–15, 21–22) chromosomes of man. These regions form associations in metaphase and this has been interpreted as a remnant of nucleolar activity. The association formed by these acrocentric chromosomes is of medical interest because these chromosomes are often involved in trisomies and reciprocal translocations (Yunis, 1974). As far as it is known, nucleolar organizers have the rDNA spaced by heterochromatin. The reasons for this structural organization are not known but heterochromatin seems to be involved in the relative preservation of ribosomal cistrons from evolutionary changes (Yunis and Yasmineh, 1971; Sutton, 1972; Chapter 3).

Unlike bacteria, the 5 S ribosomal cistrons are not closely linked to the 18 and 28 S cistrons in eukaryotes. In man, for example, these genes (2000 copies per haploid genome; Hatlen and Attardi, 1971) are largely but not exclusively localized at the distal end of the long arm of chromosome 1 (probably in band 1q43) (Steffensen *et al.*, 1974, 1975; Chapter 3). It is not yet known if these genes are spaced with repetitive DNA. It is known, however, that the 5 S rDNA base sequences have been highly preserved in eukaryotes (Forget and Weissman, 1967; Williamson and Brownlee, 1969; Averner and Pace, 1972). At interphase, the 5 S genes are often associated with the nucleolus in highly diverged eukaryotes (Chapter 3).

Attempts have been made to localize mRNA in *Drosophila* and in man. In *Drosophila*, poly(A) containing cytoplasmic RNA binds specifically and reproducibly to about 50 band-interband regions in the salivary gland polytene chromosomes (Spradling *et al.*, 1975). The species which were detected probably belong to the more abundant classes of RNA and large mRNA molecules. In man, using G-banded metaphase chromosomes from cultured lymphocytes and cDNA (specific activity = 6×10^7 dpm/μg) to total poly(A) messenger RNA from Wil_2 cells, a preferential distribution of labeling to light bands was observed (Yunis *et al.*, 1977) (Fig. 11).

VIII. FINE STRUCTURE OF CHROMOSOMES

The development of the quinacrine- and Giemsa-banding techniques revealed a great deal of structural differentiation along the length of mammalian metaphase chromosomes. In the human genome, 320 dark or light bands per haploid set can be observed with the standard G-banding technique. Each dark/light band would therefore have an average of about 200 structural genes if we assume that man has 30,000 structural genes. In contrast to this, in the case of the

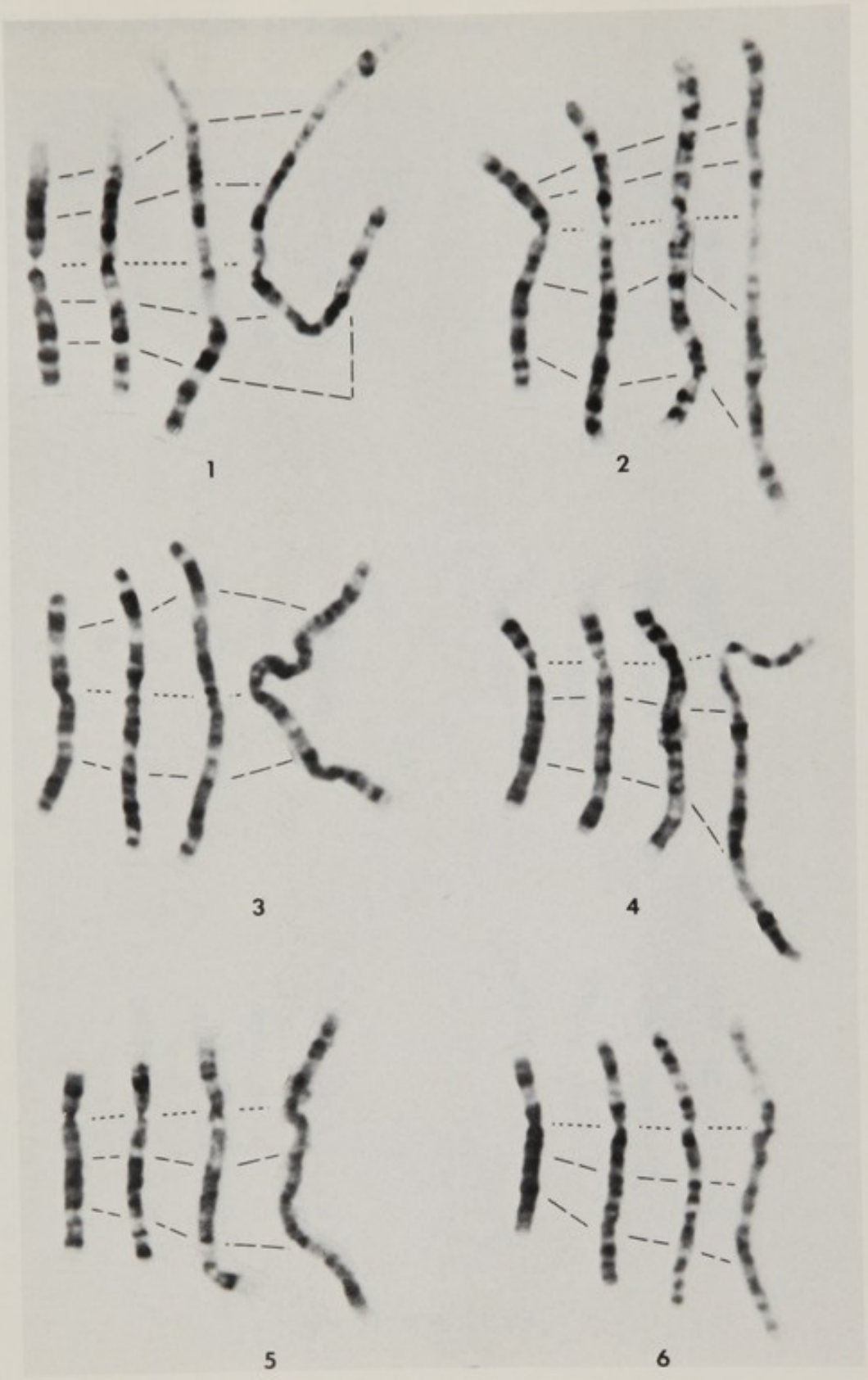

Fig. 12. Illustration of G-banded human chromosomes at different stages of mitosis. From left to right, each chromosome is represented at midmetaphase, early metaphase, early prometaphase, and late prophase, respectively. Note the progressive coalescence of the multiple fine bands of late prophase into the thicker and fewer dark and light bands of metaphase (*continued*). (From J. J. Yunis, unpublished.)

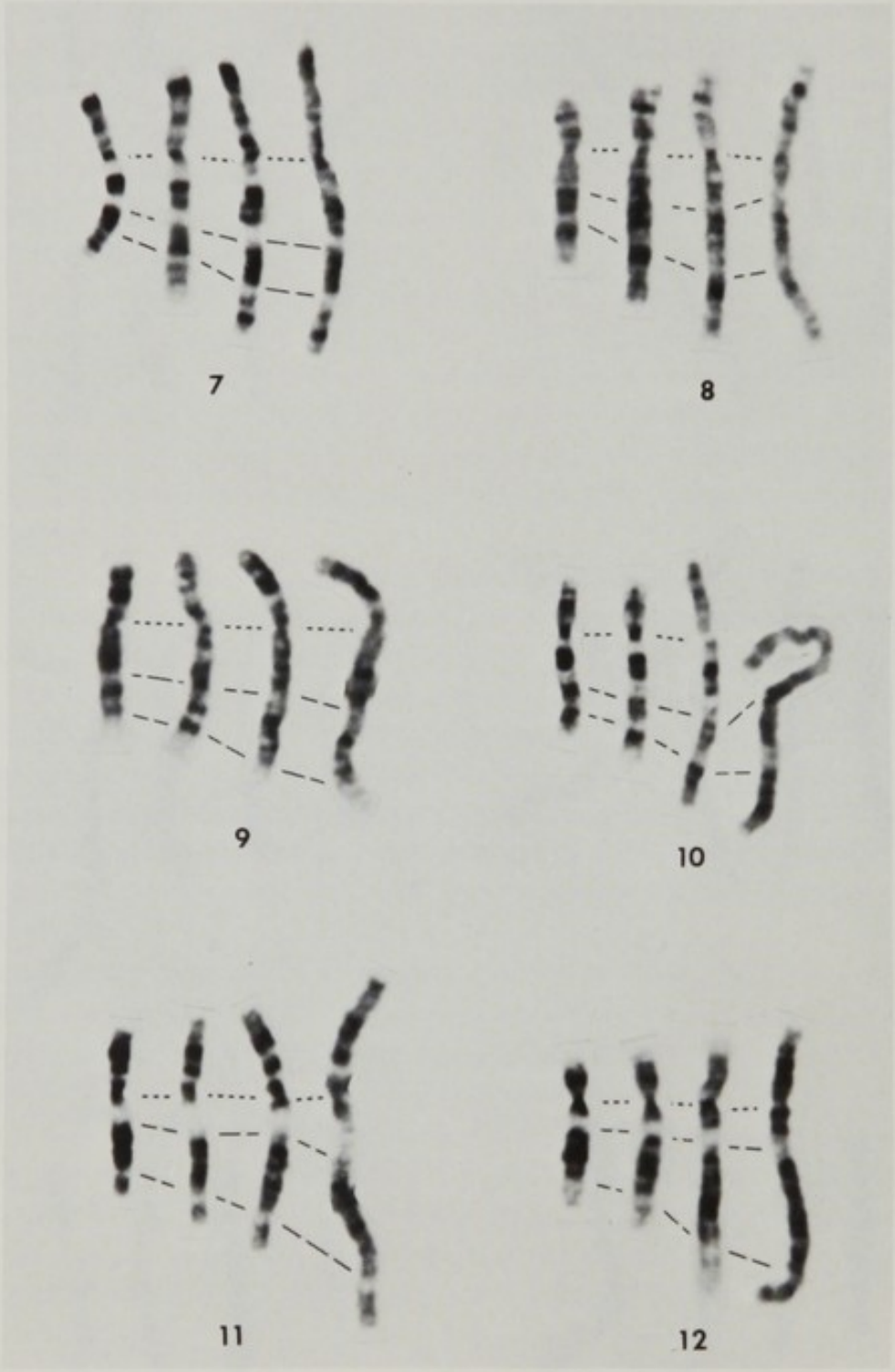

Fig. 12. *(continued)*

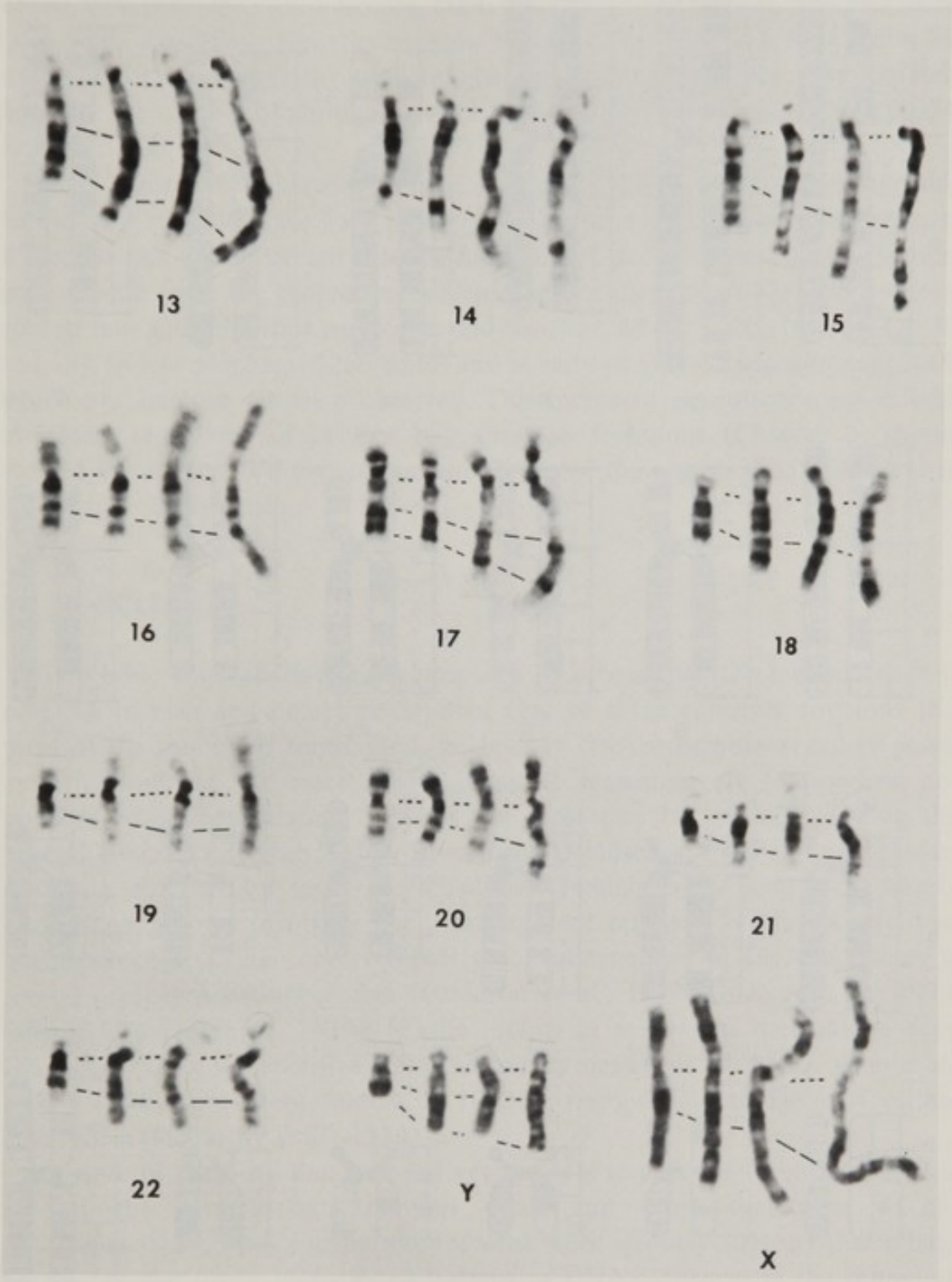

Fig. 12. *(continued)*

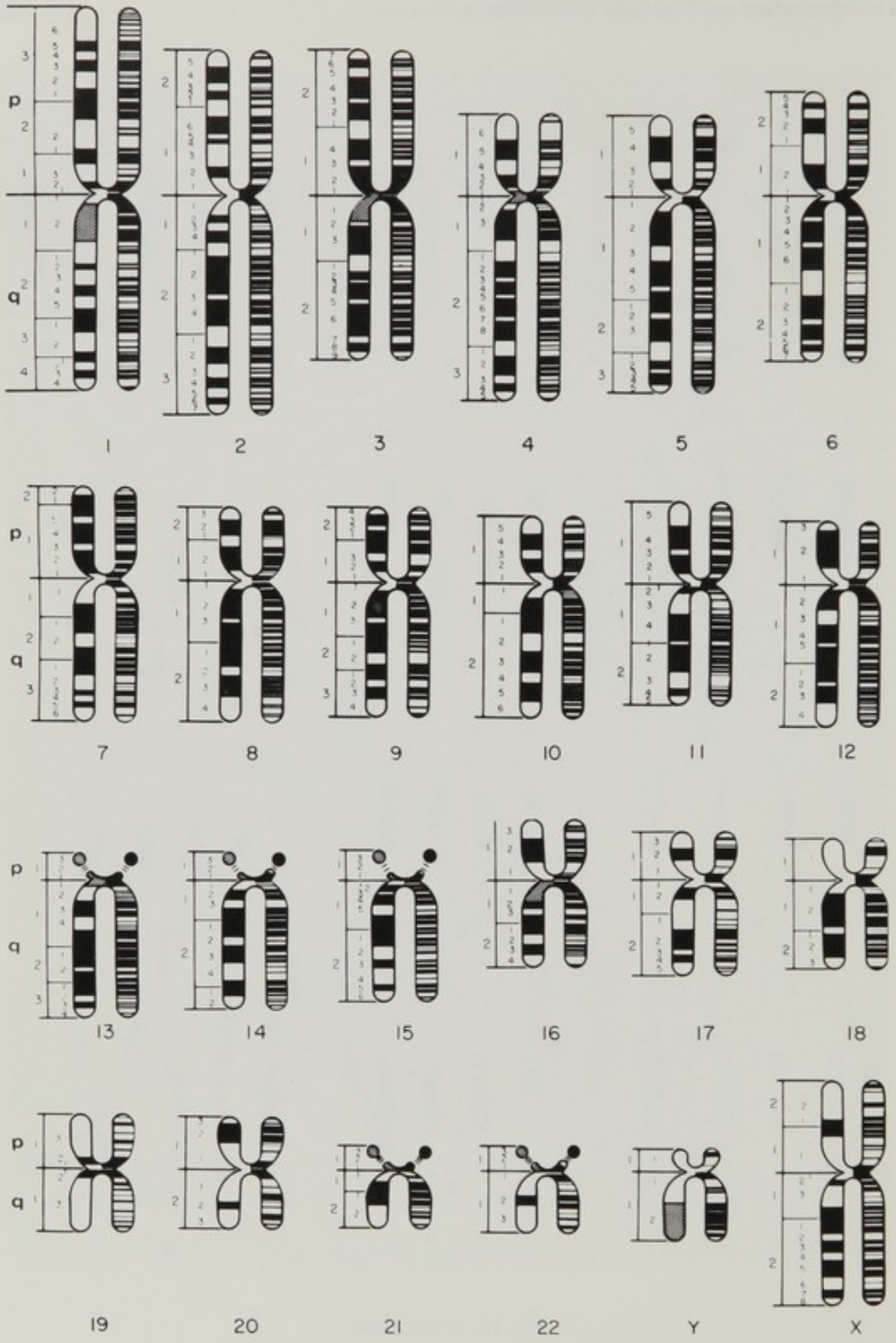

Fig. 13. Comparative representation of human chromosomes following Paris Conference Nomenclature. In each chromosome, left chromatid represents banding pattern observed in midmetaphase, while the right chromatid represents G-banding pattern observed in late prophase. (From Yunis, 1976.)

Drosophila polytene chromosomes, 5000 band/interband regions (chromomeres) have been identified. Genetic analysis based on the observed total inducible mutations suggests that the total number of structural genes is approximately equal to the number of chromomeres (Judd *et al.*, 1972; Judd and Young, 1974; LeFevre, 1974).

In an attempt to achieve with human chromosomes a resolution comparable to that of *Drosophila*, we have recently developed a simple G-banded prophase technique and demonstrated that the major bands of human metaphase chromosomes result from the progressive coalescence of numerous smaller bands uncovered at late and midprophase (Yunis and Sanchez, 1975; Yunis, 1976) (Figs. 12 and 13). In late prophase, 1256 bands and in early-prophase approximately 3000 bands per haploid set were observed. This increased resolution coupled with improved sensitivity of *in situ* hybridization technique (Chapter 3) should expand the ability to integrate our knowledge of the organization of DNA with cytological observations.

IX. CONCLUSION

Two basic concepts have been proposed to account for the known "excess" of DNA in man and higher eukaryotes. One of these concepts envisions that most of the genome is transcribed, single copy DNA represents structural genes, and the bulk of the intermediate repeated sequences are interspersed and involved in gene regulation. Perhaps the most popular model engulfing this general concept was proposed by Britten and Davidson in 1969 and modified by Davidson and Britten in 1973. Persuasive arguments in favor of a largely transcribed genome involving finely interspersed repeated and nonrepeated sequences include (1) the observed pattern of interspersion of DNA sequences in several organisms including man (Davidson *et al.*, 1973; Graham *et al.*, 1974; Schmid and Deininger, 1975); (2) the finding in sea urchin that unique copy DNA contiguous to repetitive DNA transcribes messenger RNA (Davidson *et al.*, 1975); (3) the consistent finding of 10–30% transcription of DNA of various eukaryotes (McCarthy *et al.*, 1974).

In spite of these studies, it is not yet possible to account for more than 2 to 3% of the genome as structural genes and more than half of the genome as transcribed. Furthermore, recent work strongly suggests that a large portion of the finely interspersed pattern of DNA sequences in the genome is transcribed into heterogeneous nuclear RNA (Molloy *et al.*, 1974; Darnell, 1975) and may not represent the alternate pattern of structural and regulatory gene sequences originally proposed by Britten and Davidson (1969). These observations and the recent finding that the bulk of mRNA is part of HnRNA (Herman *et al.*, 1976) strengthens the long favored concept proposed by Scher-

rer, Darnell, Georgiev, and others that HnRNA represents a precursor of mRNA and is processed in the nucleus to form mRNA (Darnell, 1975).

An alternate view embodies the concept that no sharp distinction can be made between unique and repetitive DNA (Walker, 1971; McCarthy and Farquhar, 1972) and, as a corollary, a bulk of the unique copy DNA may not represent structural genes. Also it has been proposed that a significant portion of operationally defined repetitive sequences may be involved in general chromosomal organization, function, and evolution rather than in fine genetic regulation (Yunis and Yasmineh, 1971; Edström and Lambert, 1975), and that a large portion of intermediate repetitive as well as "unique" copy DNA may never be transcribed (Yunis, 1973).

Some of the most cogent arguments in favor of this general tenet include (1) the finding that approximately 10% of the genome of man and other mammals represents highly repetitive satellite DNA's that are known to be nontranscribed and largely clustered in pericentromeric and perinucleolar regions (Yunis and Yasmineh, 1971); (2) the dispensibility of 90% of the genome (including all repeated sequences) in the vegetative cells of the protozoan *Stylonychia*, suggesting that in this lower eukaryote repeated sequences are not involved in genetic regulation (Prescott and Murti, 1973; Ammerman *et al.*, 1974); (3) the absence of approximately half of the usual amount of moderately repetitive sequences (Cot 10^{-2}–1) in some microtinae (Yasmineh and Yunis, 1973); (4) the marked evolutionary differences observed among intermediate sequences in primates (Gummerson, 1972; Chapter 9) and among other closely related species (Edström and Lambert, 1975); (5) the finding in *Microtus agrestis* and in the Algerian hedgehog that the bulk of the moderately repetitive DNA of these animals is localized in large blocks of intercalary heterochromatin, presumably genetically inactive chromatin, and can be isolated as large molecular weight native DNA components in neutral cesium chloride (Yasmineh and Yunis, 1974, 1975; Willey and Yunis, 1975, 1977b); (6) the observed role of intermediate repeated sequences in nuclear membrane attachment (Franke *et al.*, 1973) and in pairing and crossing-over (Hotta and Stern, 1975).

The two proposed concepts are not mutually exclusive, and a unifying theory embodying the salient features of both will most likely emerge as new findings appear. One of the important issues that needs to be resolved before a unifying theory can be reached is the number of structural genes that exist in mammals. This question can probably be approached by DNA/RNA hybridization in the presence of excess messenger RNA. Additive experiments with messenger RNA isolated from different tissues at different stages of development will provide much needed information on the complexities of messenger RNA and an approximation of the number of structural genes.

The recent work of Bernardi's and Yunis' groups on the fractionation of the mammalian and human genomes into several major DNA components with

specific biased densities and varying degrees of repetitiveness (Yasmineh and Yunis, 1974; Willey and Yunis, 1975, 1977b; Sanchez and Yunis, 1976; Tsai and Yunis, 1977; Filipski *et al.*, 1973; Macaya *et al.*, 1976; Corneo *et al.*, 1975; Chapter 2) may also provide a welcome tool to help resolve the discrepancies between those investigators who believe that the bulk of the genome is involved in gene regulation and those who believe that a relatively large amount of the genome may represent blocks of nontranslational sequences which play vital roles in chromosome organization and function. Determination of the arrangement of repetitive and unique copy sequences within the individual components may reveal whether different components have different patterns of sequence arrangements, including blocks of intermediate repetitive sequences which were not found in renaturation studies of total DNA. Moreover, since the fractionated components are expected to contain more homogeneous repetitive sequences as compared to total DNA, the effect of mismatching could be minimized, thus yielding a more accurate picture. In addition, if different components show different patterns of sequence arrangement, it might be expected that they have different transcriptional potentials. This can be in part elucidated with the use of RNA-driven hybridization to examine the extent of HnRNA and mRNA represented in the different components.

A good understanding of eukaryotic genetic regulation could be achieved through a detailed characterization of HnRNA. Since at least a portion of this RNA likely represents precursors of mRNA, studies on the structure of various classes of HnRNA should yield useful insights on possible transcriptional and posttranscriptional controls in eukaryotes. The characterization of both DNA and RNA sequences should also be followed by systematic studies of their localization in high resolution chromosomes. The combined information from these studies should play a significant role in unraveling the intricacies of genetic regulation of higher eukaryotes and man.

ACKNOWLEDGMENT

Research supported in part by Grant No. HD01962 from the National Institutes of Health.

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2

DNA Sequences in Man

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I. INTRODUCTION

The organization of the eukaryotic genome is one of the major problems in molecular biology today. While our understanding of some specific facets, such as

the organization of repetitive genes (e.g. the ribosomal genes) or the nucleotide sequences of some satellite* DNA's, can be considered satisfactory, we are still far from having a clear picture of the overall problem. This also applies to the eukaryotic genome of greatest interest to us, the human genome. In fact, surprising as it may seem, we know even less about it than about some other eukaryotic genomes. The literature on the organization of the human genome is scanty and complex, if not contradictory. We have, in this article, attempted to give a detailed presentation of some specific issues, such as human satellites.

II. ANALYSIS OF THE HUMAN GENOME BY DENSITY GRADIENT CENTRIFUGATION

Since the classic work of Meselson *et al.* (1957), it has become well known that when centrifuged in CsCl density gradients, bacterial DNA's show unimodal, symmetrical bands, whereas calf thymus DNA exhibits a multimodal, asymmetrical band. A number of theoretical and experimental investigations using density-gradient equilibrium centrifugation have dealt with uniform populations of DNA molecules such as phage DNA's (Schmid and Hearst, 1969, 1971, 1972). Early attempts to study the much more complex animal DNA's (Sueoka, 1959, 1961, 1962) have not been followed up.

Research in this field was stimulated by the work of Filipski *et al.* (1973) that showed that the CsCl main band of calf DNA, MW = $5–7 \times 10^6$, was not formed by genome fragments having a continuous distribution of their average G + C content, as was commonly believed, but by three distinct families of fragments. These were identified by their buoyant densities in CsCl and were equal to 1.697, 1.704, and 1.709 gm/cm^3, representing about 50, 25, and 10% of the DNA, respectively. These DNA components could be separated from one another by preparative centrifugation in Cs_2SO_4-Ag^+ density gradients. They were characterized by symmetrical bands that exhibited standard deviations close to those of bacterial DNA's of comparable molecular weight. These components were responsible for the asymmetry of the CsCl main band of calf DNA.

Three important questions raised by the existence of the components of main band DNA from calf concerned their evolutionary significance, their relative arrangement in the genome, and their intramolecular heterogeneity. These problems were investigated by Thiery *et al.* (1976) and by Macaya *et al.* (1976). These authors investigated the DNA's from twenty-five eukaryotes by density gradient centrifugation; a smaller number of DNA's were studied after degrada-

*For the sake of convenience, we will call the simple-sequence or highly repetitive DNA's satellite DNA's.

tion with restriction enzymes. For the sake of clarity, a brief outline of the experimental approach used will be presented first.

A. The Experimental Approach

The approach involved the following steps:

1. DNA's, characterized by their sedimentation properties and G + C contents, were centrifuged to equilibrium in CsCl, and their modal and mean buoyant densities, ρ_0 and $\langle\rho\rangle$, were determined. The buoyant density, ρ, at any distance r from the rotation axis, can be calculated using Eq. (1),

$$\rho = \rho_\kappa - \frac{\omega^2}{2\beta_0}(r_\kappa^2 - r^2) \tag{1}$$

where the subscript κ refers to a marker DNA, ω is the angular velocity in radians sec^{-1}, and β_0 is taken as equal to 1.19×10^9 $cm^5 gm^{-1} sec^{-2}$ (Ifft *et al.*, 1961). Under such conditions, using phage 2C DNA (ρ = 1.742 gm/cm^3; Szybalski, 1968) as a density marker, a reproducible modal buoyant density, ρ_0 (density at the peak maximum, located at a distance r_0 from the rotation axis), of 1.7103 gm/cm^3 is obtained for *Escherichia coli* DNA.

The mean buoyant density, $\langle\rho\rangle$, is calculated from the first moment of the band profile about the center of rotation [Eq. (2)],

$$\langle r\rangle = \frac{\int_0^\infty cr\,dr}{\int_0^\infty c\,dr} \tag{2}$$

and from Eq. (1), c being the DNA concentration at point of abscissa r.

2. DNA's were fractionated by preparative Cs_2SO_4-Ag^+ density gradient centrifugation. Owing to differential binding of Ag^+ ions, such a procedure can resolve not only a number of apparent and cryptic satellite DNA's (Corneo *et al.*, 1968b), but also the discrete components forming the main band of calf thymus DNA (Filipski *et al.*, 1973).

Since the resolution of DNA components in Cs_2SO_4-Ag^+ density gradient centrifugation depends on pH and r ratio (the molar Ag^+/DNA-P ratio), preliminary experiments were done, usually in the analytical ultracentrifuge, to assess the influence of these two parameters on the band profiles of the DNA's investigated. The ranges explored were 7.8–10.3 for pH and 0.2–0.4 for the r ratio. Figure 1 shows the profiles of human placenta DNA obtained in the analytical ultracentrifuge in Cs_2SO_4-Ag^+ density gradients at two pH's. Since Ag^+ has an overall preferential affinity for GC base pairs at pH <8 and for AT base pairs at pH >8, the skewness of the profiles in Fig. 1 changes between pH 7.8 and pH 9.2. At pH 7.8, the G + C-rich molecules will be heavier than the A + T-rich ones, due to preferential Ag^+ binding (Jensen and Davidson, 1966);

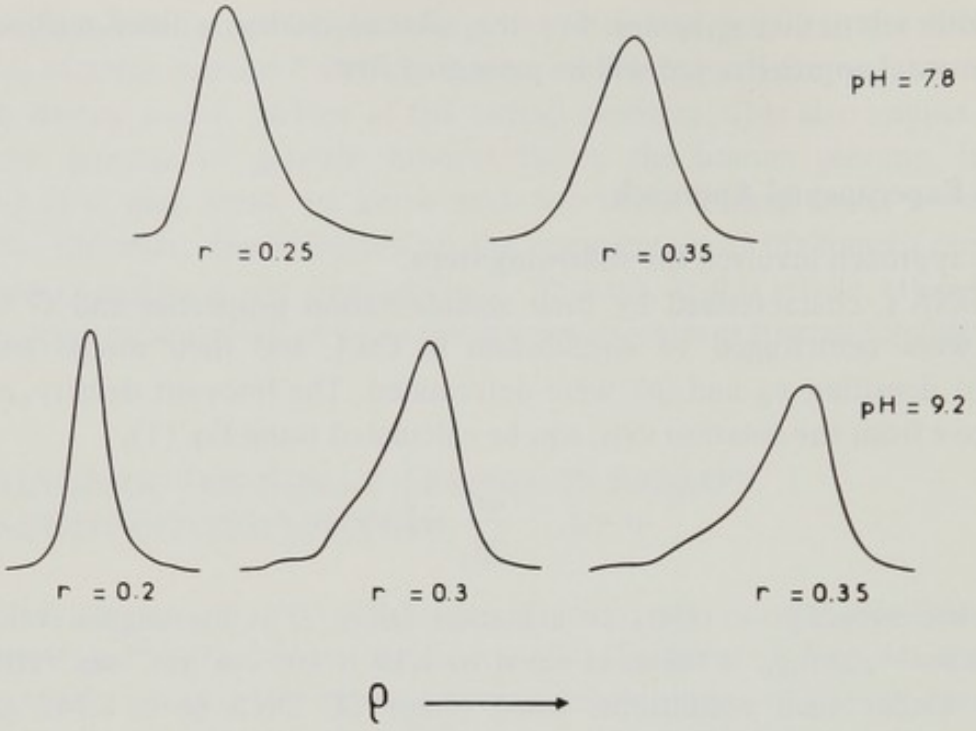

Fig. 1. Human placenta DNA in Cs_2SO_4-Ag^+ analytical density gradients; 0.04 A_{260} units of DNA in 0.005 *M* borate buffer were centrifuged, at the pH and *r* ratio indicated, for 24 hours at 44,000 rpm and 25°C in a Beckman Model E analytical ultracentrifuge, equipped with a photoelectric scanner, multiplexer, and mirror optics. The bands are shown in an arbitrary density scale, only for band profile comparison.

thus the skewness of the peak is toward high buoyant densities. At pH 9.2, the skewness is toward low buoyant densities, due to preferential Ag^+ binding by A + T-rich molecules. At pH 9.2 and $r = 0.30$ and 0.35 (Fig. 1), two satellites are apparent on the light side of the profile. These satellites will be discussed later (see Fig. 2, fractions 10–13) in preparative Cs_2SO_4-Ag^+ density gradients. Experimental conditions (pH, *r*, slope of the Cs_2SO_4 density gradient, load) giving the maximum spread and skewness of the main band were chosen; for human placenta DNA, they were pH 9.2 and $r = 0.30$–0.35.

After equilibrium centrifugation in preparative Cs_2SO_4-Ag^+ density gradients, the 15–25 fractions obtained from total DNA were centrifuged to equilibrium in analytical CsCl density gradients and analyzed. The band profiles were resolved into a number of Gaussian curves and the corresponding DNA components were defined in terms of their buoyant densities and relative amounts in the DNA's analyzed. Resolution of the CsCl profiles was done on a DuPont de Nemours (Wilmington, Delaware) curve resolver model 310. The criteria used to obtain such resolution are given elsewhere (Thiery *et al.*, 1976).

3. On the basis of the experience acquired in step (2), direct analysis of the CsCl band profiles of unfractionated DNA was attempted, and the results satisfactorily agreed with those obtained in the more detailed investigations involving preliminary Cs_2SO_4-Ag^+ centrifugation.

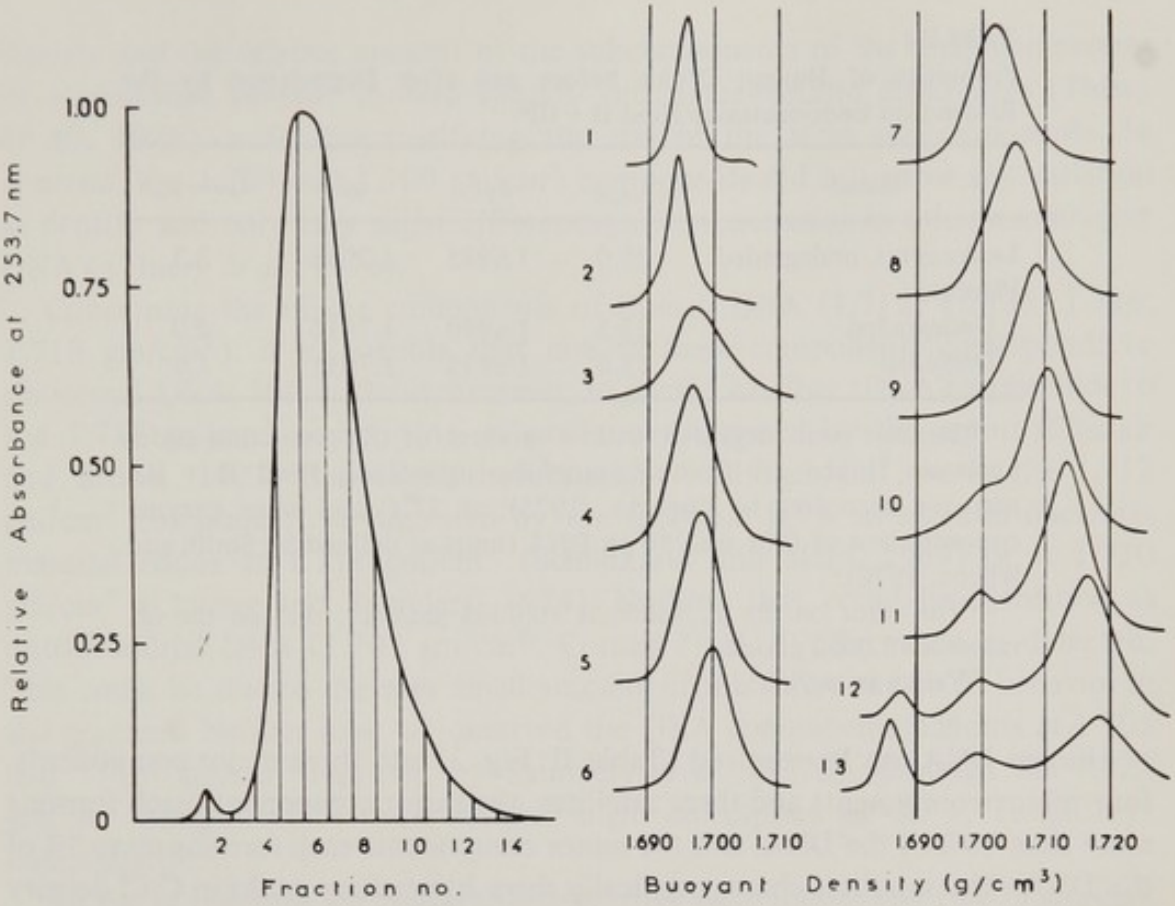

Fig. 2. Analysis of human placenta DNA components in preparative Cs_2SO_4-Ag^+ density gradient. DNA (A_{260} = 0.86) in 0.005 *M* borate buffer pH 9.2, containing $AgNO_3$ (r = 0.32) and Cs_2SO_4 (ρ_i = 1.48 gm/cm³), was centrifuged in a Beckman type 30 rotor at 25,000 rpm for 90 hours at 25°C. Fractions were analyzed in analytical CsCl gradients.

B. Results

1. Buoyant Density and Symmetry of the CsCl Band

The modal and mean buoyant densities in CsCl, ρ_0 and $\langle\rho\rangle$ for human DNA, G + C = 40.3%, are given in Table I. Table I shows the buoyant density values for Hind II + III degraded DNA (see Section II,C). The large difference between $\langle\rho\rangle$ and ρ_0, 0.002 gm/cm³, is due to the asymmetry of the peak. Such asymmetry was found in all mammalian DNA's studied by Thiery *et al.* (1976). The buoyant density differences between the DNA preparations from leukocytes and placenta are not significant.

2. Combined Cs_2SO_4-Ag^+ and CsCl Density Gradient Analysis

The greatest amount of information one can obtain from density gradient equilibrium sedimentation is derived from the combined Cs_2SO_4-Ag^+, CsCl density gradient analysis described in Section II,A,2.

TABLE I
Properties of Human DNA's before and after Degradation by the Restriction Endonucleases Hind II + III[a]

Tissue	$s_{20,w}$	ρ_0 [b]	$\langle\rho\rangle$	$\langle\rho\rangle - \rho_0$ [c]
Leukocytes, undegraded	25.0	1.6985	1.7008	2.3
Placenta				
Undegraded	22.3	1.6990	1.7010	2.0
Degraded	13.0	1.6995	1.7011	1.6

[a]Samples were degraded with a mixture of the restriction endonucleases II and III from *Haemophilus influenzae*, Hind II + III, prepared according to Kopecka (1975), at 37°C and at an enzyme concentration of 0.01 unit/50 μg DNA (units as defined by Smith and Wilcox, 1970).

[b]The error on the ρ_0 values is ±0.0005 gm/cm^3; that on the $\langle\rho\rangle$ values is ±0.0002 gm/cm^3.

[c]Values in mg/cm^3.

Human DNA can be resolved (Table II; Fig. 2) into three major components, four minor components and three satellites, the major components each forming more than 10% of the DNA, and the minor components each forming up to 3% of the DNA. The satellites characteristically show hypersharp peaks in CsCl density gradients, a feature due not only to their well-known degree of homogeneity, but also to concatenation phenomena (Macaya *et al.*, 1976).

The three major components of human placenta DNA consist of (1) a main component forming 65% of the DNA; this component is formed by two subcomponents, banding at 1.697 and 1.699 gm/cm^3 and forming 16% and 49% of the genome, respectively; (2) two major components banding at 1.704 and 1.709 gm/cm^3 and forming 18 and 11% of the DNA, respectively.

The three major components have been detected, by the same procedure, in the DNA's of three more mammals: calf, guinea pig, and mouse. The buoyant

TABLE II
Component Pattern of Human Placenta DNA[a]

Components					
Major		Minor		Satellites	
ρ	%	ρ	%	ρ	%
1.697	16	1.712	0.7	1.6875	0.15
1.699	49	1.7135	1.5	1.6955	1.2
1.704	18	1.716	1.3	1.6995	0.5
1.709	11	1.718	0.3		

[a]Combined Cs_2SO_4-Ag^+ and CsCl analysis.

density and the relative amount of the subcomponents of the main component of mammalian genome showed species- and tissue-specific differences (Thiery *et al.*, 1976), a finding justifying the use of the term subcomponents. In contrast, the 1.704 and 1.709 gm/cm^3 components did not show any variation in density and only very slight differences in concentration in other mammalian DNA's (Thiery *et al.*, 1976).

Concerning the minor components of human DNA (1.712, 1.7135, 1.716, 1.718 gm/cm^3), it is possible that one of these components corresponds to ribosomal DNA. It is difficult, however, to decide whether rDNA corresponds to the 1.718 gm/cm^3 component, as would be suggested by the report (Sinclair and Brown, 1971) that rRNA hybridizes at 1.719 gm/cm^3, or to the 1.712 gm/cm^3 component, as suggested by the fact that DNA enriched in nucleolar material bands at 1.713 gm/cm^3 (Schildkraut and Maio, 1969) or at 1.710 gm/cm^3 (Chuang and Saunders, 1974). Nothing that could be identified as mitochondrial DNA (1.707 gm/cm^3, Corneo *et al.*, 1968a) could be detected. This could be due to the very small amount of this DNA and to its behavior in the gradient. Neither have we observed the DNA components banding at 1.703 and 1.726 gm/cm^3 reported by Saunders *et al.* (1975), although the 1.720 gm/cm^3 component of these authors might correspond to our 1.718 gm/cm^3 component.

So far as satellites are concerned, only three were found: the 1.6875 gm/cm^3 (Fig. 2, fractions 12,13), 1.6955 gm/cm^3 (Fig. 2, fractions 1,2), and the 1.6995

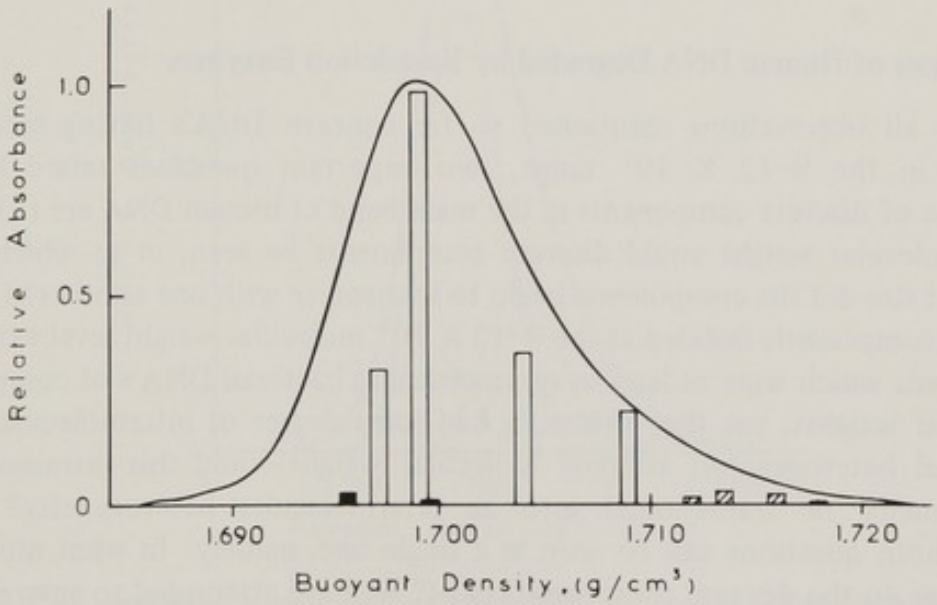

Fig. 3. A histogram of the DNA components of the human genome. The height of the bars is proportional to the percentage of each component, their width is arbitrary; empty bars correspond to main components, hatched and solid bars correspond to minor and "satellite" components, respectively. The CsCl band profile of this DNA is shown superimposed on the histogram.

gm/cm^3 (Fig. 2, fractions 10–13). A detailed discussion of human satellite DNA's will be given in Section III.

A histogram of the DNA components of the human genome, superimposed on the CsCl profile of this DNA is shown in Fig. 3.

3. CsCl Analysis

Thiery *et al.* (1976) have shown that direct resolution of the DNA components analyzed above was possible with fairly satisfactory precision, so far as buoyant densities and relative amounts of DNA are concerned, by using the CsCl profiles of unfractionated DNA. This approach is evidently much less laborious than the Cs_2SO_4-Ag^+ density gradient fractionation followed by CsCl band analysis of the fractions.

The results obtained with two different preparations of human DNA, from placenta and leukocytes, are shown in Table III; Fig. 4 shows the CsCl analysis of placenta DNA. The component pattern obtained for placenta DNA is in good agreement with that obtained by the more elaborate analysis presented in Section II,B,2, with the expected exception of minor and satellite components. An interesting finding is that different relative amounts of the two subcomponents were observed in DNA preparations from two different tissues; in contrast, the 1.704 and 1.709 gm/cm^3 are present in equivalent amounts in the DNA from both sources.

C. Analysis of Human DNA Degraded by Restriction Enzymes

Since all observations mentioned so far concern DNA's having molecular weights in the 9–12 $\times$ 10^6 range, two important questions raised by the existence of discrete components in the main band of human DNA are (1) Up to what molecular weight could discrete components be seen, or at which DNA fragment size did the components begin to intersperse with one another? (2) The discrete components isolated at the 9–12 $\times$ 10^6 molecular weight level exhibited CsCl bands which were at least as symmetrical as bacterial DNA's of comparable molecular weights, yet they evidently had some degree of intramolecular compositional heterogeneity; at what molecular weight could this intramolecular heterogeneity be transformed into an intermolecular heterogeneity? Quite clearly both questions can be seen as a single one, namely: In what molecular size range do the discrete components exist? We have attempted to answer these questions by investigating, in terms of buoyant densities and relative component amounts, human DNA degraded to a molecular weight of 2 $\times$ 10^6 by the restriction enzymes from *Haemophilus influenzae* Hind II and III.

TABLE III
Component Patterns of Human DNA's[a]

DNA source	Major components		Minor components and satellites	
	ρ	%	ρ	%
Leukocytes	1.697	27	1.693	3
	1.699	39	1.713	2
	1.704	19		
	1.709	9		
Placenta	1.697	13	1.692	3
	1.699	52	1.716	1
	1.704	19		
	1.709	11		

[a]CsCl analysis.

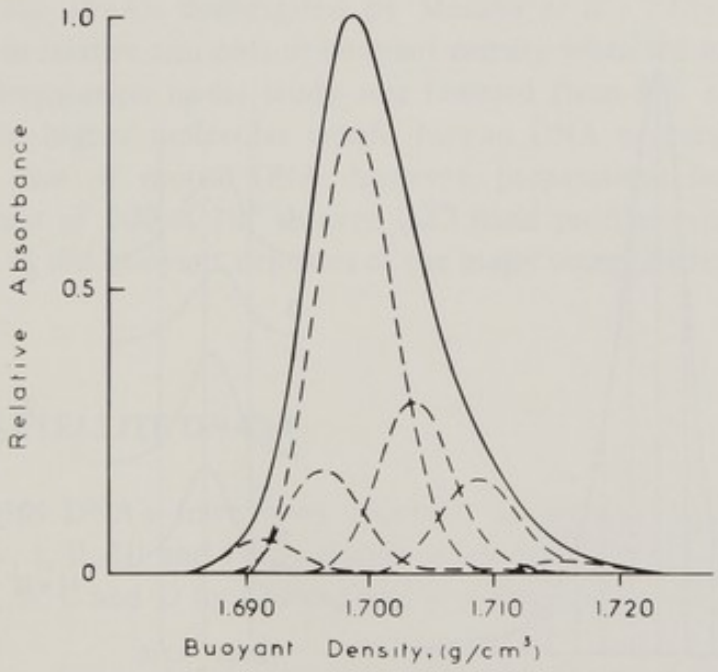

Fig. 4. CsCl analysis of human placenta DNA; 0.05 A_{260} units of DNA in 0.01 *M* Tris, 0.005 *M* NaCl pH 7.6 were centrifuged for 24 hours at 44,000 rpm and 25°C in the analytical ultracentrifuge. The optical density profile so obtained was analyzed in terms of component curves using a DuPont 310 curve resolver.

1. Buoyant Density and Symmetry of the CsCl Band

Table I shows the sedimentation coefficient, modal and mean buoyant densities in CsCl, ρ_0 and $\langle\rho\rangle$, and the $\langle\rho\rangle - \rho_0$ values for Hind II + III degraded human placenta DNA. The ρ_0 values of undegraded and degraded samples are exactly the same within accepted experimental error; the $\langle\rho\rangle - \rho_0$ values reflects the error in ρ_0.

2. Fractionation in Preparative Cs_2SO_4-Ag^+ Density Gradients and CsCl Analysis of the Fractions

Figure 5 shows the results obtained for human placenta DNA; the component pattern is shown in Table IV. A comparison of the data in this table with those in Table II shows that the major components of the degraded DNA were similar, in both buoyant density and relative amounts, to those of the undegraded samples; slight but significant density shifts were found for the 1.697, 1.704, and 1.709 gm/cm^3 components.

So far as minor components are concerned, the 1.716 gm/cm^3 component does not seem to change in buoyant density and relative amount. The 1.712, 1.7135, and 1.718 gm/cm^3 components cannot be recognized anymore, and a new minor component, 1.695 gm/cm^3, appears after degradation.

As for the satellite DNA's, two of them were not degraded or only very

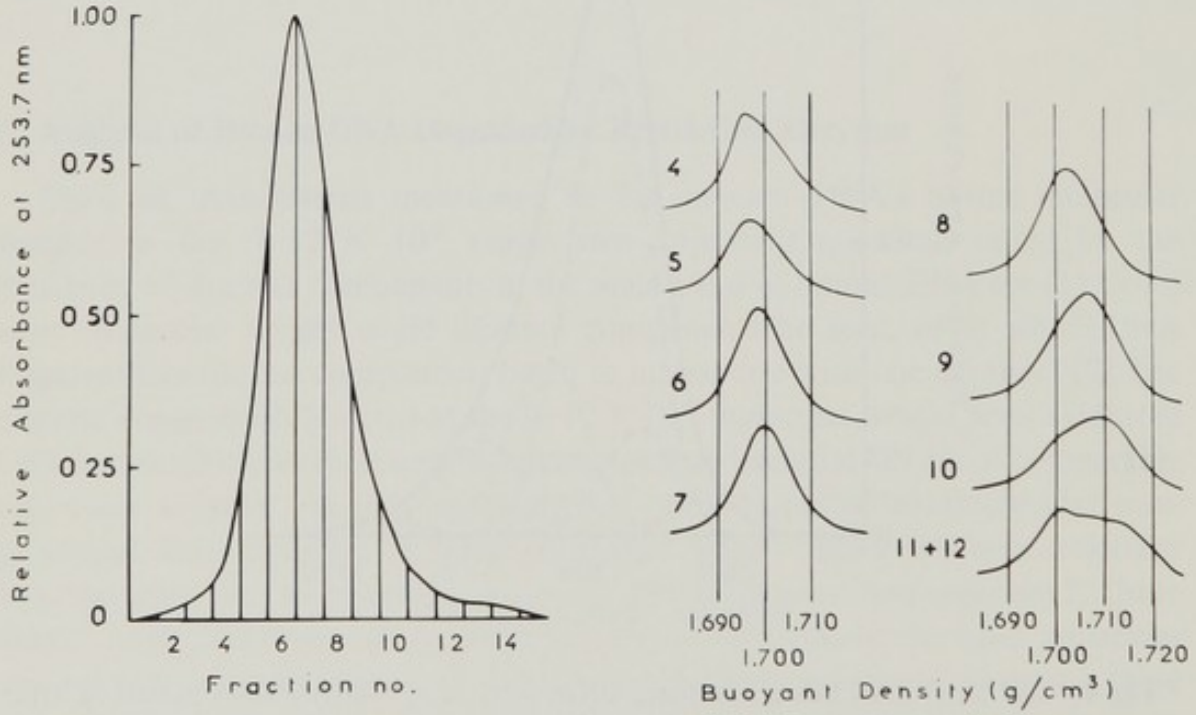

Fig. 5. Analysis of human placenta DNA components in preparative Cs_2SO_4-Ag^+ density gradient after degradation by Hind II + III. DNA (A_{260} = 0.49) in 0.005 *M* borate buffer pH = 9.2, containing $AgNO_3$ (r = 0.32) and Cs_2SO_4 (ρ_i = 1.50 gm/cm^3), was centrifuged in a Beckman type 65 rotor at 35,000 rpm for 64 hours at 25°C.

TABLE IV
Component Pattern of Human Placenta DNA Degraded by the Restriction Enzymes Hind II + III[a]

Components					
Major		Minor		Satellites	
ρ	%	ρ	%	ρ	%
1.6965	16	1.695	2.5	1.695	1.2
1.699	46	1.716	1	1.700	0.5
1.7045	19				
1.710	9				

[a]Combined Cs_2SO_4-Ag^+ and CsCl analysis.

partially degraded (1.695 and 1.700 gm/cm^3, Fig. 5, fractions 4 and 11 + 12) as shown by the appearance of the corresponding bands; the 1.6875 gm/cm^3 satellite was probably degraded and was difficult to recognize as a satellite in the digested DNA (Fig. 5, fractions 11 + 12, compared with Fig. 2, fractions 12 and 13).

In conclusion, the major components of human DNA (as well as those of the other mammalian DNA's investigated by Macaya *et al.*, 1976) did not show major changes in relative amounts or buoyant density when the molecular weight of the DNA preparation under study was lowered from 9×10^6 to 2×10^6. Experiments on higher molecular weight human DNA preparations were not done. In the case of mouse DNA, however, preparations having molecular weights in excess of 200×10^6 showed CsCl band profiles exhibiting maxima corresponding to the buoyant densities of the major components (Macaya *et al.*, 1976).

III. HUMAN SATELLITE DNA's

Eight satellite DNA's have been reported in human DNA: four, named chronologically, I, II, III and IV by Corneo *et al.* (1967, 1970, 1971, 1972) and four named A, B, C and D by Chuang and Saunders (1974) and Saunders *et al.* (1972b, 1975).

Table V shows some of the properties reported for these satellite DNA's. The situation now seems so complex that a detailed discussion on each satellite will be given separately, but some general comments appear to be useful at this point.

Not all of the satellites reported to date were isolated by the same procedure and from the same DNA sample. Usually three different methods were used:

TABLE V
Properties of Human DNA Satellites

Satellite	ρNeut. CsCl	ρAlk. CsCl	ρRean. CsCl	% Total	Separation properties	% G + C	T_m	References[d]
I	1.687	1.707 1.738	1.697	0.5	Heavy side Cs_2SO_4-Hg^{2+}, $r = 0.1$, pH = 9.2	26.1[a] 27.5[b]	80°C in 1× SSC	1,2
					Light side Cs_2SO_4-Ag^+, $r = 0.2$, pH = 9.2, separated at $r < 0.2$			3
					Late eluted from MAK; Light side Cs_2SO_4-Ag^+, $r = 0.23$, pH = 9.2			4
	1.686	1.731 1.764		0.2		26.4[c]		5
	1.687	1.732 1.765						6
II	1.693	1.740 1.750	1.696	2.0	Heavy side Cs_2SO_4-Ag^+, $r = 0.35$, pH = 9.2	43.2[a] 33.7[b]	87°C in 1× SSC	7
					Heavy side Cs_2SO_4-Ag^+, $r = 0.20$, pH = 9.2; separation increases with r, not separated at $r < 0.2$; Early eluted from MAK			3 4
	1.695					34[b]		6
III	1.696	1.740 1.754	1.703		Light side Cs_2SO_4-Ag^+, $r = 0.2$, pH = 9.2; separated at $r < 0.2$.	35.9[a] 36.7[b]	84°C in 1× SSC	3
				1.5	Late eluted from MAK; light side Cs_2SO_4-Ag^+, $r = 0.23$, pH = 9.2			4
				2–3	Light side Cs_2SO_4-Hg^{2+}, $r = 0.1$, pH = 9.2			8
	1.699	1.759 1.772						6

IV	1.700	1.730 1.742	1.706	2	Light side Cs_2SO_4-Ag^+, r = 0.23, pH = 9.2; early eluted from MAK	41[b]		4
A	1.710	1.775	1.715	0.5–1	Light side Cs_2SO_4-Ag^+, $r > 0.2$, pH = 9.2; separation increases with r	52[a] 51[b]	88.2°C in 0.18 M Na^+	9
	1.712					53.8[c]		5
B	1.726				Light side Cs_2SO_4-Ag^+, pH = 9.2; lighter than A			9
	1.726	1.792		<0.5	(Ribosomal genes)	67[b]		10
C	1.703	1.760	1.712		Light side Cs_2SO_4-Ag^+, r = 0.27, pH = 9.2	44[b]	72.9°C in 0.025 M Tris-HCl	11
D	1.720	1.782				61[b]		6
"1.687"	1.6875			0.15	Light side Cs_2SO_4-Ag^+, r = 0.32, pH = 9.2			12
"1.695"	1.6955			1.2	Heavy side Cs_2SO_4-Ag^+, r = 0.32, pH = 9.2			12
"1.700"	1.6995			0.5	Light side Cs_2SO_4-Ag^+, r = 0.32, pH = 9.2			12

[a] Base composition calculated from the melting temperature (T_m) according to Marmur and Doty (1962).
[b] Base composition calculated from the buoyant density (ρ) according to Schildkraut *et al.* (1962).
[c] Base composition measured by direct chemical analysis.
[d] References:

1. Corneo *et al.* (1967).
2. Corneo *et al.* (1968a).
3. Corneo *et al.* (1971).
4. Corneo *et al.* (1972).
5. Schildkraut and Maio (1969).
6. Saunders *et al.* (1975).
7. Corneo *et al.* (1970).
8. Mitchell (1974).
9. Chuang and Saunders (1974).
10. Chuang (1974).
11. Saunders *et al.* (1972a).
12. Thiery *et al.* (1976).

(1) CsCl density gradient centrifugation (Corneo *et al.*, 1967; Schildkraut and Maio, 1969); (2) Cs_2SO_4-Ag^+ density gradient centrifugation (Corneo *et al.*, 1970, 1971, 1972; Chuang, 1974; Chuang and Saunders, 1974; Gosden and Mitchell, 1975; Saunders *et al.*, 1975); and (3) Cs_2SO_4-Hg^{2+} density gradient centrifugation (Corneo *et al.*, 1967, 1970). These were used on total or on previously fractionated DNA via nucleoli preparations (Chuang, 1974; Schildkraut and Maio, 1969); chromatin fractions (Corneo *et al.*, 1971; Gosden and Mitchell, 1975); MAK column chromatography fractions (Corneo *et al.*, 1972); and "Cot fractions" (Hearst *et al.*, 1973; Saunders *et al.*, 1972b).

The general properties of the different satellites are given in Table V. In the following discussion, we have tried to collate the results and compare them with our own. Basically, a "satellite" DNA is characterized according to its density in neutral CsCl, in alkaline CsCl where strand separation can be analyzed, and in neutral CsCl after denaturation and renaturation under defined conditions. Base composition, when measured analytically, will be useful in this characterization, particularly when compared with values calculated from the melting temperature (T_m) or from the buoyant density. Intramolecular heterogeneity of the isolated satellites can be analyzed by thermal denaturation and reassociation analysis.

A. Satellite I

First isolated by Corneo *et al.* (1967), this satellite has been found by several other authors (Saunders *et al.*, 1975; Schildkraut and Maio, 1969; Thiery *et al.*, 1976). It exhibits a buoyant density of 1.687 gm/cm^3 in neutral CsCl and strand separation in alkaline CsCl; the density difference between the two strands is about 33 mg/cm^3. The absolute ρ values of the strands in alkaline CsCl as obtained by different authors are difficult to compare because of differences in the methods used to calculate the buoyant density in these experiments. This satellite is found on the heavy side of the main band in a Cs_2SO_4-Hg^{2+} density gradient, at pH 9.2 and $r = 0.1$ (Corneo *et al.*, 1967, 1968a); in Cs_2SO_4-Ag^+ density gradients it is found on the light side of the main band, at pH 9.2, being well separated at $r < 0.2$ (Corneo *et al.*, 1971). In agreement with these results, we found this satellite on the light side of the main band in a Cs_2SO_4-Ag^+ density gradient at pH 9.2 and $r = 0.32$ (Fig. 2, fractions 12 and 13). According to our data, this satellite accounts for 0.15% of the genome, a value lower than the 0.5% reported by Corneo *et al.* (1967, 1968a, 1971, 1972), but of the same order as that of 0.2% given by Schildkraut and Maio (1969) or 0.15–0.175% calculated from hybridization experiments by Jones *et al.* (1974) and Moar *et al.* (1975). Base compositions calculated from ρ_0 and T_m are in good agreement with the analytical value of 26.4% G + C content found by Schildkraut and Maio (1969).

B. Satellite II

This satellite DNA, first found by Corneo *et al.* (1970), has a density in neutral CsCl of 1.693 gm/cm^3 and shows strand separation in alkaline CsCl; the density difference between the strands is 10 mg/cm^3. This satellite, found on the heavy side of the main band in Cs_2SO_4-Ag^+ density gradients at pH 9.2, is separated at $r > 0.2$, the separation increasing with r (Corneo *et al.*, 1970, 1971). Corneo *et al.* (1971) reported a T_m of 87°C in 1X SSC, but Moar *et al.* (1975) report a value of 84°C in the same solvent. G + C contents of 43.2% and 35.9% can be calculated from T_m values of 87°C and 84°C, respectively. using the relationship of Marmur and Doty (1962); a G + C content of 33.7% can be calculated from ρ = 1.693 gm/cm^3, using the relationship of Schildkraut *et al.* (1962). In the analysis of human placenta DNA using the combined Cs_2SO_4-Ag^+ and CsCl method, we did not find, on either side of the main band, a satellite with a density of 1.693 gm/cm^3. On the heavy side of the main band, at pH = 9.2 and r = 0.32, we found a satellite DNA similar in that respect to satellite II, but with a buoyant density of 1.6955 gm/cm^3 in neutral CsCl. This density value is very close to that reported by Corneo *et al.* (1971) for their satellite III (see Section III,C). Saunders *et al.* (1975) found a density of 1.695 gm/cm^3 for human satellite II. It is thus very difficult with the data available to decide if our 1.6955 gm/cm^3 satellite is equivalent to satellite II. Corneo *et al.* (1970) gave as 2% the amount of this satellite in the genome, but by hybridization experiments Moar *et al.* (1975) found a value of 0.7%; we found 1.2% for our 1.6955 gm/cm^3 satellite in human placenta DNA.

C. Satellite III

First described by Corneo *et al.* (1971), this satellite has a buoyant density in neutral CsCl of 1.696 gm/cm^3; the two strands separate in alkaline CsCl with a buoyant density difference of 14 mg/cm^3 (Corneo *et al.*, 1971). This satellite is found on the light side of the main band in a Cs_2SO_4-Ag^+ density gradient at pH 9.2 and r = 0.2 (Corneo *et al.*, 1971, 1972). Saunders *et al.* (1975) found a buoyant density of 1.699 gm/cm^3 for what they called satellite III, and a difference in buoyant density of the separated strands in alkaline CsCl of 13 mg/cm^3. In this respect, these data are closer to those for satellite IV (see Section III,D). Our 1.6955 gm/cm^3 satellite DNA, even if it is found on the opposite side of the main band in a Cs_2SO_4-Ag^+ density gradient with respect to satellite III, has a buoyant density and amount (1.2%) closer to that reported for satellite III (1.5% Corneo *et al.*, 1972). Moar *et al.* (1975) found that satellite III accounts for 0.85% of the genome using hybridization experiments. Base compo-

sitions calculated from T_m (84°C, Corneo *et al.*, 1971) and ρ are in good agreement, 35.9% and 36.7% G + C, respectively (Marmur and Doty, 1962; Schildkraut *et al.*, 1962).

D. Satellite IV

Found by Corneo *et al.* (1972) after MAK fractionation of human DNA, satellite IV has a buoyant density of 1.700 gm/cm^3 in neutral CsCl and exhibits strand separation in alkaline CsCl, with a density difference of 12 mg/cm^3. It accounts for 2% of the genome (Corneo *et al.*, 1972). Our 1.6995 gm/cm^3 satellite DNA is present in a lower amount, 0.5%, but, as for satellite IV (Corneo *et al.*, 1972), it is found on the light side of the main band in a Cs_2SO_4-Ag^+ density gradient at pH 9.2 and $r = 0.32$ (see Fig. 2, fractions 10–13).

E. Satellite A

By CsCl density centrifugation of HeLa nucleolar DNA, Schildkraut and Maio (1969) fractionated a component having a buoyant density in neutral CsCl of 1.712 gm/cm^3. This component can be seen as a satellite band in CsCl band profiles of total nucleolar DNA preparations. In alkaline CsCl it does not show strand separation; its analytical base composition is 53.8% G + C (Schildkraut and Maio, 1969). Chuang and Saunders (1974) have isolated, by centrifuging human nucleolar DNA in Cs_2SO_4-Ag^+ density gradient at pH 9.2, a satellite with a density in neutral CsCl of 1.710 gm/cm^3; its buoyant density in alkaline CsCl is 1.775 gm/cm^3 and there is no strand separation (Chuang and Saunders, 1974). Renaturation analysis of this satellite (see Section V,B) shows a biphasic renaturation curve with a fast reassociating component accounting for 22% of the total (Chuang and Saunders, 1974). Schildkraut and Maio (1969) reported that, on heating and rapid cooling, their 1.712 gm/cm^3 heavy satellite shows in CsCl two bands of equal area (1.727 and 1.716 gm/cm^3). Each of these bands had the base composition of the double stranded DNA; thus they did not consist of complementary strands separated as a result of heating. On the contrary, after denaturation-renaturation, Chuang and Saunders (1974) found a single band at 1.715 gm/cm^3. If there are close similarities between the two heavy satellites described by Schildkraut and Maio (1969) and Chuang and Saunders (1974), more work should be done to establish their identity.

F. Satellite B

This satellite, described by Chuang and Saunders (1974) is found as a small shoulder on the light side of satellite A when it is prepared in a Cs_2SO_4-Ag^+ density gradient at pH 9.2 and $r = 0.40$. It has a buoyant density of 1.726

gm/cm^3 in neutral CsCl. Chuang and Saunders (1974) claim that this satellite DNA has many properties consistent with a DNA having ribosomal genes and ribosomal spacers. *In situ* hybridization experiments with cDNA prepared from this satellite show heavy grains clustered at the nucleoli. When unlabeled 18 S + 28 S rRNA was used as a competitor for the *in situ* experiments, all grains in the nucleoli disappeared.

G. Satellites C and D

Not much data is available for these two satellites isolated by Saunders *et al.* (1972a, 1975). Satellite C can be seen as a satellite band on the light side of a Cs_2SO_4-Ag^+ density gradient at pH 9.2 and $r = 0.27$; it has a buoyant density of 1.703 gm/cm^3 in neutral CsCl. For satellite D, see Table V.

In view of the facts just described, and with the data available, it is difficult to correlate our findings on human satellite DNA's with those of other authors. Of the eight satellites studied, four of them, I to IV, are best characterized. Ambiguities in data exist however, between satellites II and III: for example, Saunders *et al.* (1975) reported densities of 1.695 gm/cm^3 and 1.699 gm/cm^3 for satellites II and III; these values are nearer to the values given by Corneo *et al.* (1971, 1972) for their satellites III and IV; the density difference in alkaline CsCl for the separated strands of satellite III reported by Saunders *et al.* (1975), 13 mg/cm^3, is as close to the difference found by Corneo *et al.*, (1972) for their satellite IV, 12 mg/cm^3 as to that for their satellite III, 14 mg/cm^3 (Corneo *et al.*, 1972). Our analysis of human placenta DNA showed the existence of three satellite DNA's of densities in neutral CsCl of 1.6875, 1.6955, and 1.6995 gm/cm^3 (see above). Our 1.6955 gm/cm^3 satellite reinforces the ambiguity between satellites II and III: it has a buoyant density close to that found by Corneo *et al.* (1971, 1972) for their satellite III but it is found on the heavy side of the main band in Cs_2SO_4-Ag^+ density gradients at pH = 9.2 and $r = 0.32$ as is satellite II (Corneo *et al.*, 1970). Melli *et al.* (1975) have shown that there is cross-hybridization between satellites II and III. All this may indicate that these two satellites are either closely related or are the same satellite.

For satellites A, B, C, and D more work should be done for their identification. No strand separation has been observed in any of these satellites; their concentration is not well-defined in total DNA. Satellite B has been associated with ribosomal genes by Chuang and Saunders (1974), even if its buoyant density seems too high in view of the findings of Sinclair and Brown (1971) that *Xenopus* ribosomal RNA hybridizes with HeLa cells DNA at a density of 1.719 gm/cm^3. Satellites C and D show some specificity in *in situ* hybridization (see Section IV), a fact that can argue for their human origin, against the contaminant DNA argument. Satellite A shows no specific hybridization, all chromosomes being labeled.

IV. *IN SITU* HYBRIDIZATION

A. Satellite DNA

Results of *in situ* hybridization of human satellite sequences reported by various authors are contradictory. Moar *et al.* (1975) have recently shown that in *in situ* hybridization experiments, the dispersion of the autoradiographic label throughout the karyotype is dependent on the incubation temperature. As a consequence, all the results of *in situ* hybridization of human satellite se-

TABLE VI
Chromosomal Localization of Human Satellite DNA (*in Situ* Hybridization)

Satellite	Chromosomal localization		References[a]
	Main	Other	
I	Y		1
	Y	1,3,13,14,16,21	2
	Y,9	14,15,21,22	3
II	1	9,16	4
	Y	9	1
	Y,9	1,15,16,17,21,22	3
III	9	D and G groups, 1,16	5
	Y		1
	9,15,Y	13,14,20–22	3
		1,7–11,13–22,Y[b]	6
IV	Y		1
	9,15,Y	13,14,20,21,22	3
A	Not localized		7
B	Nucleoli		8
C	9	D and G groups	9
D	9		10

[a]References:
1. Evans *et al.* (1974)
2. Jones *et al.* (1974)
3. Gosden *et al.* (1975)
4. Jones and Corneo (1971)
5. Jones *et al.* (1973)
6. Moar *et al.* (1975)
7. Chuang and Saunders (1974)
8. Chuang (1974)
9. Saunders *et al.* (1972a)
10. Saunders *et al.* (1975).
11. Tanguay *et al.* (1975).

[b]No distinction was made between main and other localizations.

quences should be revised. For example, Jones *et al.* (1973) reported that cRNA prepared from human satellite III hybridizes *in situ* mainly to chromosome 9 and to a lesser extent to chromosomes of the D and G groups. Moar *et al.* (1975) reported for this same satellite III definite sites of hybridization not reported by Jones *et al.* (1973); labeling near the centromere is found in chromosomes 1, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and Y.

Table VI shows the chromosomal localization reported for human satellite sequences. These results clearly show the existing confusion. Chromosomes 1 and 9 show systematic labeling with cRNA prepared from 6 of the 8 satellites studied. Chromosome Y shows labeling with 4 of them. In the human karyotype, the largest areas of heterochromatin are found in chromosomes 1, 9, and 16 (and Y) (Arrighi and Hsu, 1971; Yunis *et al.*, 1971; Yunis and Yasmineh, 1972).

B. Repetitive DNA

Only *in situ* hybridization experiments done with cRNA prepared from "Cot fractions" will be discussed here.

By *in situ* hybridization experiments, Saunders *et al.* (1972b) have shown that repetitive DNA is species-specific. A similar result was found by Hearst *et al.* (1973) with "h.a.r.r. DNA" (hydroxyapatite-isolated rapidly renaturing DNA). Interpretation of these results should be made with great care, since the specificity of hybridization may come from satellite sequences preferentially transcribed from repetitive DNA during cRNA preparation. Using two "Cot-fractions", human fast repetitious DNA (Cot 0–0.05) and intermediate repetitious DNA (Cot 0.05–50), Saunders *et al.* (1972b), found that both of them are species-specific. Satellite sequences are expected to be enriched in the "fast repetitious" fraction, but in view of the peculiar renaturation properties of some human satellite DNA (see Section V,B), some satellite sequences are probably found in the "intermediate repetitious" DNA. Using cRNA prepared from two "h.a.r.r. DNA" fractions, 1.703 and 1.714 gm/cm^3, Hearst *et al.* (1973) found species-specific hybridization in *in situ* experiments; this "h.a.r.r. DNA" preparation was free of at least three of the known satellites.

V. RENATURATION PROPERTIES OF HUMAN DNA

A. Total DNA

Renaturation experiments done on a number of highly sheared animal DNA's have shown practically constant proportions of repetitive and nonrepetitive or unique sequence DNA's (Davidson *et al.*, 1975); unique sequences usually

account for 60–75% of the genome. The repetitive sequences can be subdivided into two classes: the fast repetitive sequences (a fraction that usually includes the highly repetitive or satellite sequences) and the intermediate or middle-repetitive sequences. A third class of sequences, the "foldback" or inverted sequences, have not been extensively studied, although they have been identified in some organisms (Hearst *et al.*, 1973; Wilson and Thomas, 1974; Schmid *et al.*, 1975; Schmid and Deininger, 1975).

Renaturation studies on the human genome have shown that the relative amounts of repeated and unique sequences are the same as for other animal DNA's, as expected. Saunders *et al.* (1972b) reported 35% repetitive and 65% unique sequence DNA; essentially the same figures were found by Mitchell (1974) and Schmid and Deininger (1975).

Even if the relative amounts of repeated and unique sequence DNA's are fairly constant in different animals, the arrangement of these sequences within the genome seems to follow two different patterns of organization, the so-called *Xenopus* and *Drosophila* patterns (Davidson *et al.*, 1975). The "*Xenopus* pattern" is characterized by the interspersion of single copy sequence segments of lengths from 800 to several thousand base pairs, with repetitive sequence segments about 300 base pairs long (Davidson *et al.*, 1973, 1974). The "*Drosophila* pattern" is characterized by the existence of single copy sequence elements about 40,000 base pairs long with no interruptions by repetitive sequences; the middle repetitive sequences (nonsatellite sequences) are of an average length of 5000 base pairs (Manning *et al.*, 1975).

Saunders *et al.* (1975) have reported that about 10% of human DNA is repetitive, arranged as repetitive sequence segments, 1100 base pairs long, and interspersed with less repetitive sequences longer than 2500 base pairs.

Schmid and Deininger (1975) studied the sequence organization of the human genome. Basically, the human genome follows the "*Xenopus* pattern". Repetitive sequence classes are distributed throughout 80% of the genome. About one-half of the genome is made up of short single copy sequences, 2000 base pairs long, interspersed with very short repetitive sequences of an average length of 400 base pairs. A second fraction of the genome is composed of an interspersed arrangement of very long unique sequences with short repeated sequences.

The organization of the inverted sequences in human DNA has been studied by Hearst *et al.* (1973), Wilson and Thomas (1974), and Schmid and Deininger (1975). About 3% of the genome can be isolated as "foldback" DNA (Hearst *et al.*, 1973). Wilson and Thomas (1974) have shown that these inverted sequences ("palindromes") are found in clusters of two to four, these clusters being sparsely distributed; the palindromes are not randomly located, specific sequences being adjacent to them. Schmid and Deininger (1975) suggested that the inverted repeat sequences should not be considered a subclass of repeated

sequences. According to these authors, inverted repeat, repetitious, and single copy sequences are mutually interspersed. The arrangement of repeated and single copy sequences near an inverted repeat is similar to the arrangement found elsewhere in the genome.

A different approach to the study of the sequence organization in the human genome had been used by several authors. This approach consists of the study of the buoyant properties in CsCl of renatured DNA fragments, previously fractionated according to their renaturation properties ("Cot fractionation"), thermal stability, or MAK column chromatography (Saunders *et al.*, 1972b; Hearst *et al.*, 1973; Corneo *et al.*, 1975). This kind of work allows the isolation of different classes of molecules among a "Cot" family; some of these classes of molecules can be associated with satellite DNA (Hearst *et al.*, 1973), others reflect the true nonsatellite repetitious molecules.

B. Satellite DNA

Renaturation data on human satellite DNA is scarce. Chuang and Saunders (1974) studied the renaturation kinetics of human satellite A, sonicated to an average length of 250 nucleotides. The renaturation curve was biphasic: 22% of the DNA had an apparent $Cot_{1/2}$ of 1.3×10^{-2} and 54% an apparent $Cot_{1/2}$ of 8.3. Thus the first component can be classified in the highly repetitive and the second one as an intermediate repetitive DNA, the fast component being 1600 times more frequently repeated than the slow component. This satellite was found to be intermolecularly homogeneous by the author. If that is the case, the renaturation kinetics reflect a particular intramolecular heterogeneity.

At least three of the five classes of human "h.a.r.r." DNA molecules studied by Hearst *et al.* (1973) can be associated with satellite DNA. For two of them, 1.687 and 1.700 gm/cm^3, renaturation analysis was done, showing a biphasic renaturation curve, the first one with 32% of a kinetic complexity of 20 base pairs and 68% with a kinetic complexity of 530 base pairs; for the second one the values are 40% of 35 base pairs and 60% of 700 base pairs.

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3

Human Gene Localization by RNA:DNA Hybridization *in Situ*

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This article is not intended to be a complete review of gene localization by *in situ* hybridization, but rather a restricted coverage involving the human genome. Since very few loci have been located directly, it is necessary to examine a variety of organisms in order to organize a strategy for the more difficult studies

in man. The theory, biochemistry, and most of the technology are now available to map every gene, but a few strictly technical problems remain. The *in situ* method of gene mapping is potentially very powerful, since in theory any gene is open to examination if the pure RNA or DNA can be isolated. So far the genes which are highly repeated have been relatively easy to locate. Definite evidence about those genes which have only one copy per haploid genome or only a few copies is much more difficult to obtain. Yesterday's technical problems seem easy now, so perhaps new advances in certain areas will make almost every gene amenable to examination. New avenues for genome analysis are in the offering. Undoubtedly there will be practical and clinical applications, which are becoming apparent even now. This review will consider today's realities and record the progress with human chromosomes and cytogenetics ending with 1975.

I. COMMENTS ON ISOLATING AND LABELING RNA

No attempt is made here to consider the detailed procedures of isolation of nucleic acids nor the techniques of getting them highly radioactive. For this specific information, the reader can consult references to be cited. The isolation methods for DNA are excluded altogether because the information is well known, as is the method for making ^{3}H-cRNA from DNA with ^{3}H-nucleotide triphosphates from DNA-dependent RNA polymerase from *E. coli* (Gall and Pardue, 1971).

There are several ways to proceed for isolating RNA from cells. The first involves cell fractionation and differential centrifugation. If the cells are relatively free of nucleases, this is the method of choice. The RNA from HeLa cells comes out relatively undegraded with cell fractionation and particle isolation methods (Knight and Darnell, 1967; Hatlen *et al.*, 1969). With small amounts of tissue, short RNA sequences are usually purified by acrylamide gel electrophoresis. To separate 5 S RNA from tRNA, 8 or 9% gels are used; discontinuous slab gels have been used to isolate individual 9 S histone messenger molecules by Grunstein *et al.* (1974). Histone messenger molecules lack poly(A) on their 3′-end, while most mRNA seem to have poly(A) added after transcription. A recent review by Greenberg (1975) covers many aspects of poly(A)-containing mRNA. The immunoprecipitation method for selectively removing a specific mRNA from polysomes by reactions involving its nascent peptides is now the select method (Schechter, 1973).

Because many sources of tissue, including human placenta, contain nucleases that degrade the RNA during fractionation and centrifugation, alternative isolation methods must be used. We have found that kilograms of tissue, fresh or frozen, can be processed directly by homogenizing in phenol:buffer mixtures using either a mortar and pestle, a motor-driven glass-to-glass homogenizer, or a

commercial size Waring blender. After the RNA is precipitated with ethanol, the large and small molecules can be separated by redissolving in 3 *M* sodium acetate. The soluble 4 S and 5 S RNA are purified further on DEAE cellulose and then separated from each other on Sephadex G-100 (Hatlen *et al.*, 1969) or on DEAE-Sephadex A-25 according to Miyazaki and Takemura (1966).

Both the 4 S and 5 S RNA are purified further by reverse phase chromatography, system 5 (RPC-5) of Pearson *et al.* (1971). Reverse phase chromatography requires both skill and biochemical experience. The best equipment is expensive and technically demanding. Ultimate purity seems to be the following: one sharp, symmetrical peak eluting from the column, resulting in one radioactive site on the chromosome when RNA is used for hybridization. Our experience with autoradiographs has given a visual display of how well or how badly the biochemistry was done. With 5 S rRNA one would observe a single labeled site with *Drosophila melanogaster* at 56F (2R) and no contamination from 18 S and 28 S RNA if the RNA was not degraded during isolation, was separated properly as mentioned above, and rechromatographed on RPC-5 after iodination. Purification on RPC-5 is especially important for high signal to low noise discrimination. With complete equipment one can get a direct optical determination of the specific activity. With human 5 S rRNA or mRNA, degradation or poor separation is obvious when the human D and G chromosomes (13–15 and 21–22) are labeled by contaminating 18 S and 28 S rRNA.

Transfer RNA is difficult to separate in pure form. So far, partial success has been obtained with *Drosophila melanogaster*, where the very large salivary chromosomes are used. Our first effort (Steffensen and Wimber, 1971) gave an indication that it was feasible to map tRNA. We used tRNA which had been labeled *in vivo* with ^{3}H-uridine. In our hands, BD-cellulose did not give satisfactory separation, so we could never be certain of having a pure iso acceptor. With the advent of iodination of RNA (Prensky *et al.*, 1973), it was possible to do biochemistry on large amounts of RNA *before labeling* so the tRNA could be purified thoroughly. After all criteria for a single isoacceptor were fulfilled, the molecule could be iodinated. Even one of the most experienced laboratories found it difficult to remove 5 S RNA contamination from tRNA, as indicated when the ^{125}I-RNA was hybridized to *Drosophila* chromosomes. According to Grigliatti *et al.* (1974) purified $tRNA_5^{LYS}$ binds at 48F–49A on 2R, and yet there was quite a bit of 5 S rRNA contamination binding to 56F. Szabo (1974) succeeded in purifying both tRNA and 5 S RNA, so it has been possible to obtain the ideal "one peak, one label site" with *Drosophila* chromosomes without having to resort to competition with cold RNA. In most cases, however, apparently pure tRNA peaks on RPC-5 are not really pure but often contain 2 or 3 molecular species that show up on autoradiographs. Since tRNA is modified by various additions to bases after transcription, the same molecular species can exhibit changes which alter chromatographic properties.

The 18 + 28 S RNA can be purified a number of ways: (1) sucrose gradients; (2) CF 11 cellulose column (Franklin, 1966); (3) hydroxyapatite; (4) MAK column (Mandell and Hershey, 1960). None of these methods are highly satisfactory for getting very pure 18 + 28 S rRNA. Fortunately, *in situ* studies with rRNA do not require highly pure rRNA because these large genes of *Drosophila* are highly repeated. If the rDNA genes are just about saturated, and hybridization is not too long, then traces of other RNA will be well below saturation and contaminating radioactivity on autoradiographs will be negligible.

Since Commerford (1971) discovered a method to iodinate nucleic acids to high specific activities, ^{125}I-RNA has been used in hybridization studies with increased frequency. The cytosines of RNA or DNA are tagged with ^{125}I. The most thorough discussion of this method has been given by Prensky (1975). There are a few important comments to add and to emphasize. Commerical vendors of ^{125}I have yet to provide a special grade for labeling nucleic acids. *Most of the difficulties* of isolating whole nucleic acid molecules at or above specific activities of 1×10^8 dpm/μg, *are due to impurities in the radioactive iodine*. The vendors should be pressured into doing better and more complete radiochemistry. Quality control for the iodination of nucleic acids should be done before they are sold. With a good batch of carrier-free iodine, RNA with specific activities of $1–6 \times 10^8$ dpm/μg can be obtained. After the first column on CF 11 cellulose (Prensky, 1975) has been run to remove unreacted iodine, then a second or third chromatographic separation should be done quickly to get rid of other impurities usually present in commercial ^{125}I. We store ^{125}I-RNA for months in 50% formamide in 2X SSC pH 6.5–6.8 with 1×10^{-4} *M* KI at −20°C. The ^{125}I-RNA seems to hybridize normally, and the silver grain yield follows the half life of ^{125}I, if the sample has been carefully repurified.

II. METHODS OF *IN SITU* HYBRIDIZATION

A. Introduction

Most *in situ* studies with vertebrate chromosomes have used the standard method of fixation in methanol and acetic acid mixture (3:1, v:v). After several changes of fixative, the cells are dropped on slides and air dried. Some of our best slides have been flame dried. Some laboratories spread metaphase cells in 66% acetic acid. Our experience is that 66% acetic acid is detrimental to the chromosomes and to hybridization efficiency. Evans *et al.* (1974a) dropped cells in 45% acetic acid onto slides supported on a hot plate. With *Drosophila* salivary chromosomes and neuroblast chromosomes, the routine procedure is to squash in 45% acetic acid, freeze the slide, and postfix in ethanol:glacial acetic acid

(3:1, v:v). We suspect that concentrations higher than 45% acetic acid must degrade the DNA and/or have drastic effects on the chromosomal proteins. For example, 66% acetic acid has proved to be the most effective way to extract ribosomal proteins completely from ribosomes of mouse liver (Sherton and Wool, 1972) and *Drosophila* (Steffensen, 1973). The removal of basic proteins from cell preparations seems to be essential to the *in situ* method. However, in order to see the chromosomes and to keep them on the slide, many proteins must be retained. At least some of these remaining proteins are probably the acidic and residual proteins that are relatively insoluble in acid. For most of the comments to follow, it is assumed that the reader is already familiar with the standard and widely used method of Gall and Pardue (1971).

In most cases it is best to hybridize slides that have been freshly prepared. Although no precise quantitative data has been worked out, experience tells us that old slides (a few months or a year old) usually give low grain counts. If preparations are stored, it is best to keep them sealed over dessicant in a deep freeze (−20°C or lower) rather than at room temperature. Part of the loss of annealing efficiency with age is due to DNA degradation and loss during hybridization. Protein denaturation occurs with age and it must have an effect. The nonhistones interfere with RNA annealing during hybridization. Methods should be devised to overcome protein blockage without loss of chromosome image. The reasons for decline of slide quality and RNA binding efficiency with time should be investigated more thoroughly. In fact, the overall efficiency must be improved by an order of magnitude before the mapping of unique sequences will be possible. Even the intermediately repeated sequence such as the tRNA genes are beyond current technology.

B. RNase

RNase is used before hybridization to remove endogenous cellular RNA so it will not compete with the labeled molecules. In several experiments the RNase step was omitted while doing ^{125}I-5 S RNA hybridization to human and *Drosophila* chromosomes (Szabo, 1974; D. M. Steffensen, unpublished). Little or no hybridization was achieved. Apparently endogenous 5 S RNA still in the preparation was sufficient to compete with the labeled 5 S RNA. This is an indirect way of testing if a particular cell type contains a particular RNA species, although direct measurements are more convincing. A concentration of RNase between 0.01–0.2 mg/μg per ml is effective, either at room temperature or at 37°C. Predigestion with RNase is not needed when cRNA made from satellite DNA of constitutive heterochromatin is used, since there is no RNA product synthesized.

C. Denaturation of Chromosomal DNA

Chromosomal DNA has been denatured three ways: (1) high temperature in a SSC solution or a SSC-formamide mixture; (2) sodium hydroxide, usually in 0.07 *N* NaOH for 30 seconds to a few minutes (Gall and Pardue, 1971); (3) acid, usually 0.2 *N* HCl for 10 to 30 minutes at 25° or 37°C. We recommend 0.2 *N* HCl for 15 minutes at 25°C. Acid gives better cytology, and the time and temperature of treatment are less critical. It is possible that 0.2 *N* HCl causes deamination of DNA. During the short time of exposure and at low temperature, any degradation with HCl that may occur does not seem to effect the binding, specificity, nor the postmelting T_m of the annealed RNA:DNA hybrids. Denaturation with alkali is used by many laboratories, and it has problems too. The precise pH and time of exposure are very critical. Slide quality with high pH denaturation is variable. After autoradiography and Giemsa staining, the NaOH-treated chromosomes are often difficult to see. Acid denaturation probably gives fewer problems and better staining. The overall methods we recommend are essentially identical to those of Dunn *et al.* (1973), derived independently.

Perhaps one reason for the superior cytology with acid denaturation is explained by the removal of remaining histones, leaving the nonhistone proteins relatively intact. The nonhistones and residual proteins are often alkali-soluble. These nonhistones maintain a semblance of chromosome structure for identification purposes.

After acid or base treatment, the slides are plunged into 0°C 2X SSC, followed by two changes of glass-distilled H_2O, dehydration in 70% ethanol, two changes of 100% ethanol, and air dried for immediate hybridization. Also the slides can go into cold 70% ethanol directly from acid or base.

In our early experiments (Wimber and Steffensen, 1970) chromosomal DNA was denatured with 95% formamide in low salt at high temperature. Although this procedure was effective, the cytology was inferior. With the same means to denature chromosomal DNA, de la Chapelle *et al.* (1973) followed the renaturation of the highly repeated DNA sequences in mouse and human chromosomes in SSC at 60°C. Double-stranded DNA complex with acridine orange fluoresces green, a clear indication of C band renaturation.

When slides for *in situ* use are quickly cooled immediately after denaturation, dehydrated in ethanol, and air dried, the chromosomes do not seem subject to DNA reannealing (Wimber and Steffensen, 1974; Szabo, 1974). The author has checked this point again by two other methods using *Drosophila* chromosomes: (1) after autoradiography, salivary chromosomes were still completely red using acridine orange fluorescence and (2) there was no loss of 1.705 gm/cm^3 DNA satellite (IV) hybridization when the slide was preincubated for 12 hours in 50% formamide in 2X SSC at 35°C *before* the ^{3}H-cRNA was added. As far as we

know, the chromosomal DNA remains immobilized on the slide in the single-stranded state. Apparently the majority of DNA is blocked from complete RNA hybridization by chromosomal protein. The whole procedure of DNA denaturation should be reinvestigated for several reasons. Formamide should be used first because it denatures DNA without using acid or base. Seventy percent formamide in 2X SSC at 60°–70° would provide denaturation conditions that are above the T_m of most DNA sequences.

D. Time, Concentration, and Temperature

Before hybridization is begun, it is necessary to make a few calculations and do preliminary experiments. Operationally, most people have used a concentration between 0.1 and 1 μg per ml with mRNA, 5 S, 18 S, and 28 S rRNA (vertebrate chromosomes) and with tRNA (*Drosophila*). The highly repeated satellite DNA, 1.705 gm/cm^3 of *Drosophila* (Peacock *et al.*, 1973; Peacock and Steffensen, 1975) will reach saturation within a few hours with ^{3}H-cRNA at 0.01 μg/ml with either salivary or mitotic chromosomes. These highly repeated sequences of 1.705 gm/cm^3 DNA are repeating units of 5-nucleotides, AAGAG (Endow *et al.*, 1975). This rapid annealing is characteristic of constitutive heterochromatin. The rate is explained by the fact that short repeats have a high probability of finding complementary strands. When the kinetics of *in situ* hybridization are compared to DNA immobilized on filters, the reaction rates are very similar (Szabo *et al.*, 1975). In both cases, the DNA is immobilized, so one can estimate from filter data about how fast or how slowly a particular RNA will anneal DNA on slides. For example, the messenger RNA for silk fibroin at 1–2 μg/ml was sufficient to reach saturation on filters at hybridization for 24–48 hours (Suzuki *et al.*, 1972). Again, the reader is reminded that this hybridization was done with formamide so that traces of ribonuclease would not degrade the messenger RNA during a long incubation period.

Recently it has been possible to quantitate the kinetics of *in situ* hybridization. Szabo *et al.* (1975) extended the work of Birnstiel *et al.* (1971) in the use of what is called a C_rt plot, an abbreviation with C_r in moles/nucleotides/liter and t in sec. Given a concentration of RNA, the time to reach saturation at the 95% level can be calculated.

E. Conditions of Hybridization

A number of workers have hybridized in varying amounts of SSC, usually between 2X SSC and 6X SSC. The salt concentration has an effect on the rate, temperature and T_m of hybridization. These methods are essentially those derived from procedures using nitrocellulose filters (Gillespie and Spiegelman,

1965). The regime we recommend for hybridization is a modification of the formamide method worked out by McConaughy *et al.* (1969). Formamide has a number of advantages: (1) Hybridization can be done at lower tempeatures where RNA is stable and does not break down. Often the conditions coincide with the temperature used for growing bacteria at 37° and 40°C. (2) The radioactive sample can be stored in mixtures of formamide and SSC in a deep-freeze without water crystals being formed. The energy from decay can thus be dissipated in liquid with less damage to other RNA molecules. (3) Ribonuclease apparently is not active in 50% formamide. It is not necessary to be as careful about sterilizing glassware and solutions when formamide is used. The RNA does not seem to degrade during hybridization. (4) The percentage of formamide can be adjusted to conditions for convenient manipulation to coincide with one's equipment, the denaturation conditions, or the T_m. A rough calculation is that 1% formamide lowers the melting point of the RNA:DNA duplex by 0.7 degrees. With 50% formamide in 2× SSC, the temperature of hybridization is lowered about 40°C. (5) ^{3}H-RNA remains stable in 50% formamide at −20°C provided the pH of the solution is at neutrality. These mixtures gave equivalent results for at least one year. Some grades of formamide contain alkaline impurities which should be avoided. Formamide made by Fluka seems to be most satisfactory both from its optical purity and neutral pH.

Recently, Robert Elder (unpublished) discovered that ^{125}I-tRNA or 5 S rRNA at a specific activity of 1×10^8 dpm/μg will stay as whole molecules and hybridize effectively for the half-life of ^{125}I (60 days) if the ^{125}I-RNA is stored in 50% formamide in 2X SSC (pH 6.8) containing 1×10^{-4} *M* KI at −20°C. The impurities from the iodination reaction are removed by reverse phase chromatography on a small RPC-5 column. In general the impurities found in the commercial ^{125}I samples and impure RNA have been responsible for the degradation of iodinated nucleic acids. Careful purification and pure reagents seem to eliminate the problems and also prolong the shelf life of the probe.

III. GENE AND GENOME EVOLUTION IN PRIMATES

A number of workers have examined the chromosomes of man and the anthropoid apes to work out homoid evolution. Pearson (1973; also Chapter 8) has data on the patterns of G-banding and C-banding which compare man, chimpanzee, gorilla, and the orangutan. The chromosomes which are most similar and show the least differences are 1, 5, 6, 13, 14, 15, 19, 20, 21, and 22. The X-chromosome is essentially identical in the four species. Annealing studies can confirm and extend banding studies, especially with 5 S RNA and chromosome 1 and with the 18 S and 28 S RNA genes on the D and G group

chromosomes. All of these chromosomes carry genes associated with ribosome function. Does the conservative nature of the latter chromosomes have anything to do with "ribosome fitness?" The difference in chromosome 1 between man and the higher anthropoids apparently involves only the addition of DNA satellite II to the long arm, a sequence unique to man (see Chapter 9). This large C-band on the long arm at band 1q12 is immediately adjacent to the centromere. The banding patterns are apparently identical for the rest of chromosome 1. Some of the earlier speculation about centric fusion between two chromosomes 12 in the chimpanzee to produce a chromosome 1 in man (Chiarelli, 1973a) must be discarded. The combined use of banding and *in situ* techniques will permit workers to understand evolution and the divergence of the primates by comprehending more fully the mechanisms of genome evolution (see Chapters 7–9).

Using R-banding to compare chromosomes 2, 7, 13, 21, and 22, Dutrillaux *et al.* (1975a) contend that *Pan troglodytes* is more closely related to *Homo* than to *P. paniscus.* A detailed band analysis is provided by Dutrillaux *et al.* (1975b) with some excellent illustration of R-, Q-, T-, and H-(C-) banding for all of the chromosomes of man and *Pongo*. Given the proper RNA probe, one could test the two inversion postulate for chromosome 7 by the latter authors in *Pongo*, converting it to the banding patterns to *Gorilla*, then to *Pan*, and finally to *Homo*.

The five pairs of acrocentric chromosomes in man (D = 13–15; G = 21–22) offer the most immediate source for advancing the understanding of primate evolution. The chimpanzee has chromosomes comparable to the human 13, 14, 21, and 22, except *Pan* chromosome 16 (human 15) does not seem to have rDNA cistrons (Henderson *et al.*, 1974b). Chimpanzee chromosome 17 has the fifth pair of chromosomes containing rDNA genes, indicative of a translocation. Similar studies must be done on the gorilla and orangutan before the rearrangements can be deciphered. Other independent methods such as cell hybridization can be used to settle these identification difficulties (Ruddle and Creagan, 1975). The rhesus macaque has a single nucleolus according to the *in situ* data (Henderson, *et al.*, 1974a), as does *Hylobates lar* (Warburton *et al.*, 1975). The nucleolar organizer chromosomes of the Old World primates have a morphology which might be recognizable in higher anthropoids (Chiarelli, 1973b). Further analysis of primate rDNA genes would seem to be a profitable way to proceed for understanding homoid evolution.

Research is being done by us to determine if the location of the 5 S RNA genes at band q43 on chromosome 1 is at the same place in the rhesus, chimpanzee, gorilla, and orangutan. Preliminary experiments indicate that the 5 S genes are, in fact, at a similar site in all four species and that this arm of chromosome 1 is highly conserved in primate evolution.

IV. RIBOSOME GENES

A. 5 S rRNA

The 5 S rRNA base sequences in eukaryotes are strictly and rigidly conserved against mutation. The 5 S RNA molecules of man, mouse, and a marsupial have identical base sequences according to Forget and Weissman (1967), Williamson and Brownlee (1969), and Averner and Pace (1972), respectively. According to Hatlen *et al.* (1969) there is a trinucleotide (ApGpG) in HeLa 5 S RNA that is not found in KB cells, which is suggestive of sequence heterogeneity in man. We do not know if this nucleotide variation represents a mutant or whether a different set of 5 S genes is expressed in KB cells from those in HeLa cells. The variation in nucleotide sequences found in normal human tissue should be examined more thoroughly in the way Brownlee *et al.* (1972) and Ford and Southern (1973) have done with *Xenopus laevis*.

The data on mapping 5 S RNA genes are not extensive enough to draw any rules about organization. 5 S RNA genes tend to be near the ends of the longer chromosomes. At interphase the 5 S genes are often associated with the nucleolus in *Xenopus* (Pardue *et al.*, 1973), *Drosophila* (Steffensen and Wimber, 1972; Szabo, 1974), Chinese hamster (Buongiorno-Nardelli and Amaldi, 1970), and human (Steffensen *et al.*, 1975). An illustration of 5 S RNA gene associations with nucleoli and with perinucleolar heterochomatic blocks is shown in Fig. 1.

No complete explanation has yet been provided to coordinate the placement of 5 S genes at interphase. A partial attempt to position the 5 S genes (Steffensen *et al.*, 1974) concerns the large C-band (DNA satellite II) on the long arm of chromosome 1 with 5 S. This block could explain the association of chromosome 1 with other segments of DNA satellite II but another binding site is needed to bring the end of the long arm to the nucleolus. A terminal or interstitial block of heterochromatin on 1q is expected to be found. Perhaps the highly repeated DNA sequences found by Hsu *et al.* (1972) at the telomere of 1q are candidates. On a hydroxyapatite column these repeated DNA molecules elute at 92°–95°C (Cot = 0–0.05) or 85°–90°C (Cot = 0.05–50) and their ^{3}H-cRNA seem to bind preferentially at the end of chromosomes 1, 2, 3, 9, and 16. The biochemistry of the latter study is given in detail by Saunders *et al.* (1972). These sequences should be further characterized.

In *Xenopus laevis* there are different 5 S rRNA sequences synthesized in kidney cells than in the ovary. These two 5 S rRNA molecules are divergent by seven base substitutions (Ford and Southern, 1973). It seems likely that a different set of genes is expressed in the kidney from those in the ovary. The 5 S RNA from *Xenopus* kidney has seven nucleotide alterations from 5 S of man, six of which are at different positions; therefore *Xenopus* ovary and human 5 S

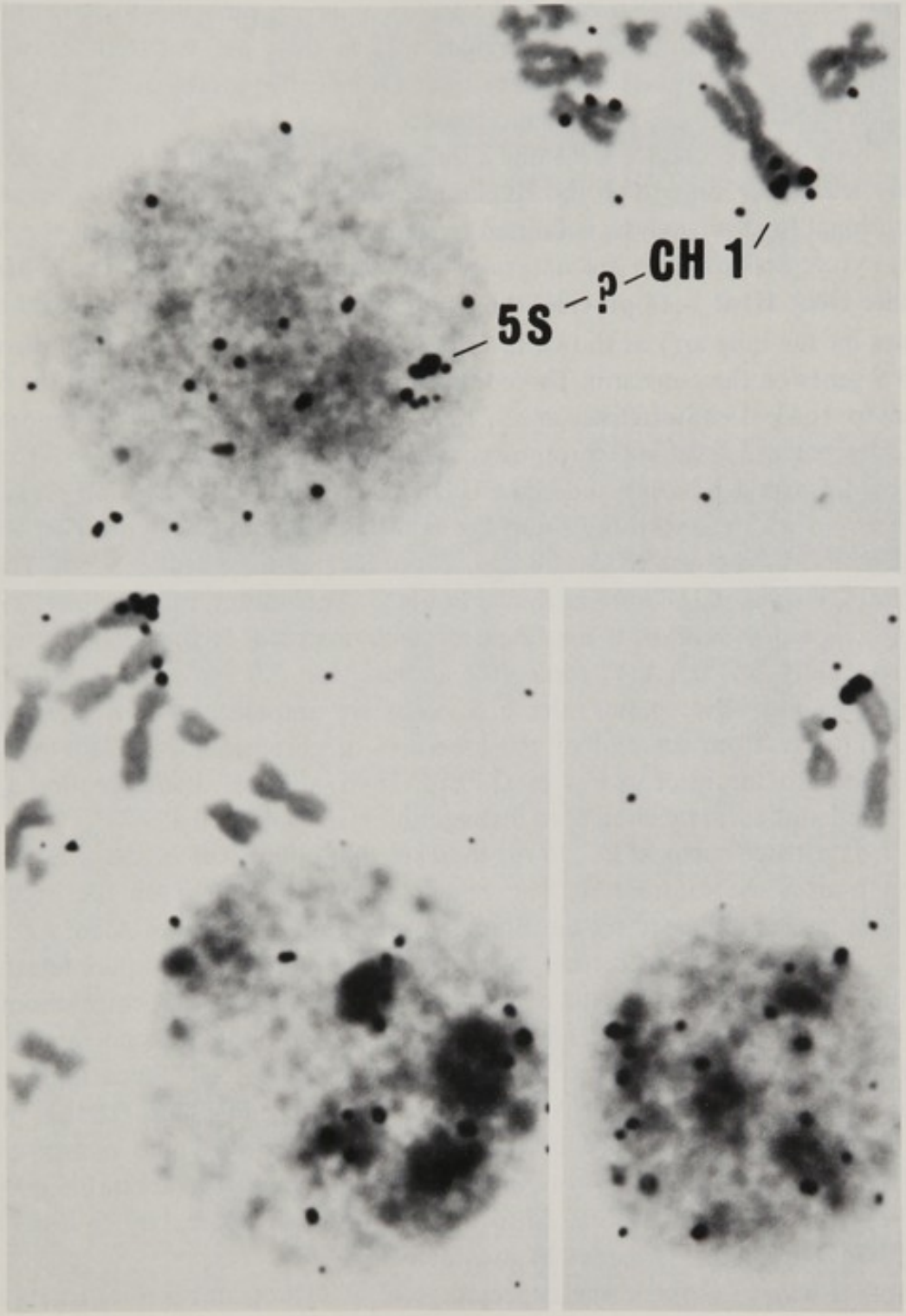

Fig. 1. Three nuclei with label from ^{125}I-5 S rRNA associated with nucleoli and perinucleolar heterochromatin. Chromosome 1 of adjacent metaphases are labeled and it is suggested that the densely labeled regions at interphase are the 5 S genes of chromosome 1.

differ by thirteen nucleotides. Since there are 5 S genes at the ends of nearly all *Xenopus* chromosomes (Pardue *et al.*, 1973), it seems amazing to have more "gene evolution" within this amphibian species than has occurred between widely divergent vertebrates. Obviously there are selective processes and adaptive advantages which we have yet to understand.

The protocol in genetics is to find a linkage group and then to map the genes linearly within the linkage group. Human genes need special techniques because conventional linkage analysis is limited by family size. In order to map the 5 S genes, cytological markers are necessary until mutants are found. The C-band (satellite DNA II) at 1q12 provides the required marker and thereby places the 5 S genes on the long arm as shown in Fig. 2. Atwood *et al.* (1975b) have placed the 5 S genes on the same arm. Three translocations were used to delimit the 5 S region to 1q41–43 (Steffensen *et al.*, 1975). McDougall *et al.* (1975) reported that adenovirus 12 caused chromosome uncoiling in the same region. These authors did a collaborative experiment. Human embryonic kidney (HEK) cells were treated with adenovirus 12 and the resulting preparations hybridized with ^{125}I-5 S rRNA. The results (Steffensen, 1976) located the uncoiler site at 1q42 and the 5 S genes at 1q42–43 as shown in Fig. 3. The resolution of iodine-125 is almost as good as tritium if the slides are underexposed. Half of the electrons have energies below 0.5 keV, while 70% are less than 3.0 keV (Prensky, 1975). With just a few silver grains, the 5 S genes are pinpointed about one grain diameter away from the end of the long arm of chromosome 1. This region corresponds to the next to last of G-band, 1q43, although the resolution and banding are not accurate enough to distinguish between 43 and 42–43.

Our data (Steffensen *et al.*, 1974) indicate that about half of the 5 S genes are on human chromosome 1. The remaining loci should be on the smaller chromosomes, as indicated by the filter hybridization evidence of Aloni *et al.* (1971). At this writing no further mapping data have appeared in the literature regarding the minor sites for the 5 S rRNA genes. We suspect that chromosomes 3, 9, 16, and maybe 22 could have such genes. However, there is no reason to accept these inferences until critical studies have been done. Since there are approximately 8000 5 S rDNA cistrons per cell at metaphase (Hatlen and Attardi, 1971), approximately 2000 copies give the 5 silver grains observed at each 1q43 in 2 months. The 4 or 5 minor gene sites would give about 0.5 grains per site, which is unsatisfactory. Until the latter number is raised by an order of magnitude, the minor 5 S genes will go undetected.

Another way to estimate the expected yield of silver grains is to calculate an expected number for chromosome 1. Given 1% ^{125}I labeling of RNA, 4% labeling of cytosine, there would be 4.8 ^{125}I atoms per 1q43 site [(2×10^3) (1.2×10^2) (0.2) (0.10) (0.01). These numbers correspond to number RNA copies × MW 5 S × autoradiographic efficiency × efficiency RNA:DNA hybridization × amount of iodination. Since just half of the iodine will decay in 2

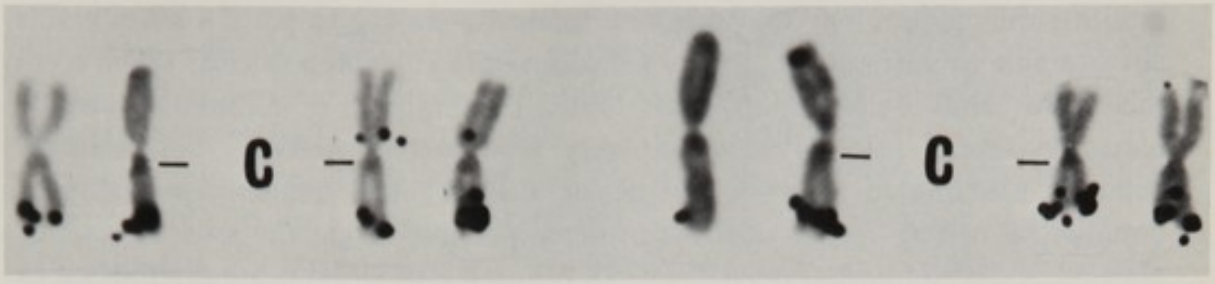

Fig. 2. Human chromosome 1 hybridized with ^{125}I-5 S rRNA. C refers to C-band at 1q12.

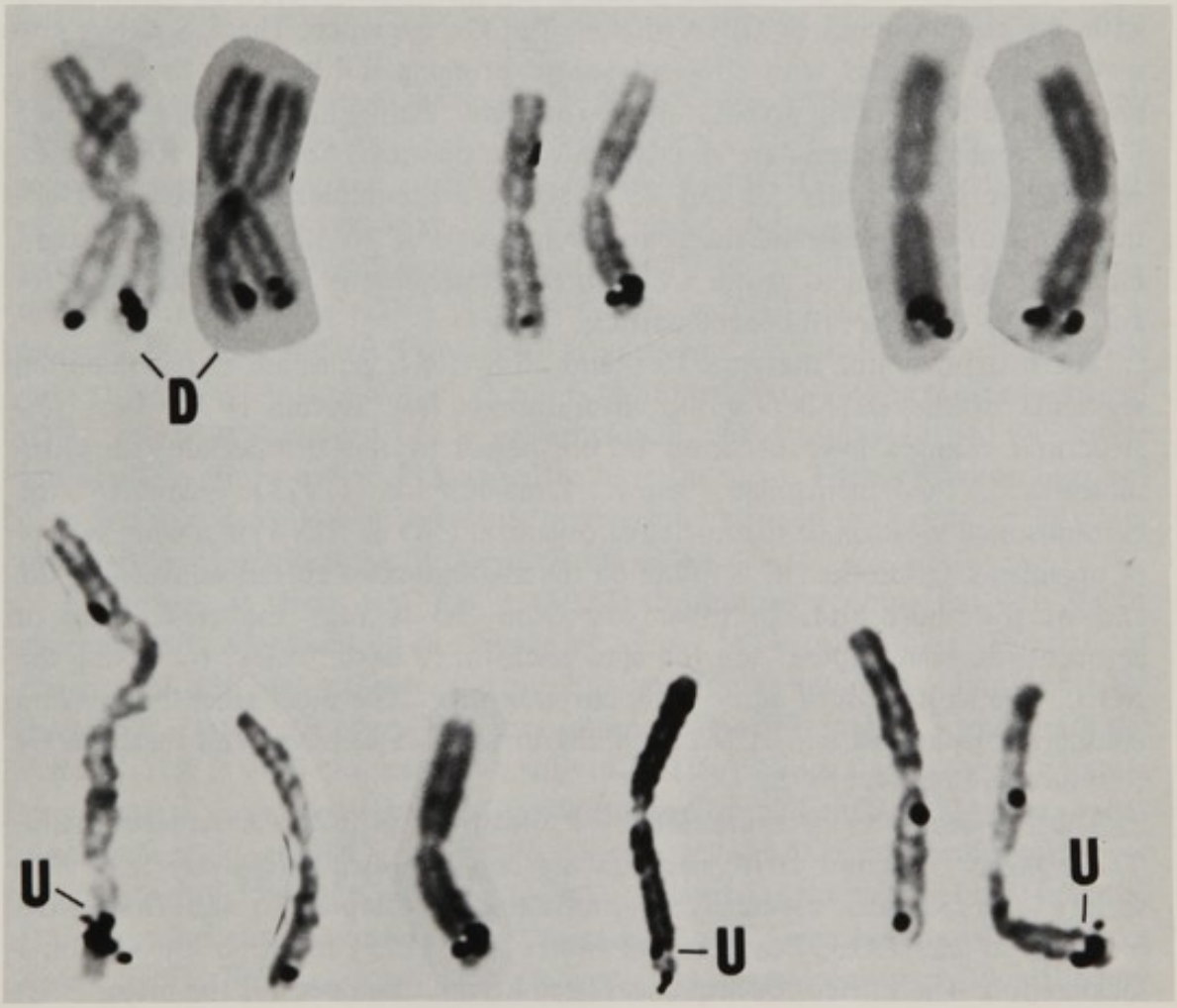

Fig. 3. *In situ* hybridization with ^{125}I-5 S rRNA to human embryonic kidney (HEK) cells treated with adenovirus 12. D, a pair of diplochromosomes; U, the uncoiling or gaps on chromosome 1 at 1q42 caused by the adenovirus 12. The virus treatment seems to bring out the banding patterns. Silver grains are most often directly over the next to last band at 1q43 or 1q42–43, immediately distal to the adenovirus uncoiler site (1q42).

months, this numerology explains how fortunate we are to get 2.4 silver grains per site and an autoradiograph from chromosome 1, let alone a clear signal from the minor sites with 10–20% less label. The amount of iodination can be increased several fold. However, at very high specific activity, the molecule becomes unstable to its energy of decay. Besides, with the uneven quality of commercial carrier-free ^{125}I, it is not often possible to get the specific activity above 1×10^8 dpm/μg (Prensky, 1975). A number of workers are attempting to improve the efficiency of hybridization with autoradiographic detection.

The molecular biology of 5 S rRNA is much more extensively known in prokaryotes. Since this molecule is universal, some of its potential functions in the larger ribosome subunit are worth describing. Fox and Woese (1975) reviewed recent data and proposed a model for the structure of 5 S RNA in prokaryotes. It is likely that 5 S rRNA (–CGAAC–) forms a specific interaction with the common arm of tRNA at the –TψCG– sequence. The 5 S rRNA also may form a complex with a few ribosomal proteins and between them form a binding site for GTPase activity on the ribosome. Unlike higher organisms, the 5 S RNA genes in bacteria are closely linked to the other ribosomal RNA genes. In *Bacillus subtilis* the 5S and 23 S rDNA are completely linked in DNA fragments of a single-strand molecular weight of 1×10^6. Apparently this tight linkage is maintained to assure a one to one stoichometry and juxtaposition for assembly of the larger ribosomal particle.

There is no doubt that the 18 S and 28 S rRNA genes are at the satellited segments of the D and G group chromosomes (see Section IV,B). Very few structural changes have occurred in the higher primates, especially for chromosome 21 as mentioned below. Lima-de-Faria (1973) examined the chromosomal location of the nucleolar organizer (NO or rDNA) in a wide variety of organisms. Often the NO is found on the medium-sized chromosomes near the end of the short arm. In other cases the NO is near the centromere of acrocentries. Man, mouse, and the apes conform to both "rules" by having the NO on the short arm of acrocentric chromosomes. The most recent studies on comparative changes and rDNA mapping in vertebrates have been reviewed by Ruddle and Creagan (1975).

The NO seems to be bracketed by a specific constitutive heterochromatin. These highly repeated DNA satellites are being studied intensively in a wide variety of organisms, especially in primates (see Chapter 9) and *Drosophila* (Peacock *et al.*, 1973; Peacock and Steffensen, 1975) for their chromosome localization. The nucleotide sequences have been determined for the major DNA satellites of *Drosophila virilis* (Gall and Atherton, 1974) and *D. melanogaster* (Endow *et al.*, 1975; Peacock *et al.*, 1973; Sederoff *et al.*, 1975). The precise base sequences for the human DNA satellites have yet to be elucidated.

In the mouse the high A:T DNA satellite at the NO forms an aggregated stalk structure that attaches to the nuclear membrane (Rae and Franke, 1972) in

Sertoli cells. It seems that fusion of identical sequences of repeated DNA at interphase will be found in most vertebrates much like the behavior of the chromocenter in *Drosophila melanogaster*. This phenomena of preferential interphase association will be discussed later.

The sequences of 18 S and 28 S RNA molecules are also highly conserved. Human rRNA forms a stable RNA-DNA hybrid with mouse nuclei at interphase and with the NO region visualized at metaphase. The T_m of rRNA/rDNA have yet to be determined for the mouse-human hybrids and the man-man hybrids. Gerbi (1972) made a wide variety of comparisons using *in situ* hybridization. The 18 S DNA (RNA) segments appear to be less likely to change in evolution than the 28 S sequence.

B. 18 S and 28 S rRNA Genes on the D and G Group Chromosomes

In the organisms which have been examined, the nucleolus organizer region (NO) always contains the genes which code for 18 S and 28 S rRNA (for reviews, see Wimber and Steffensen, 1974; Tartof, 1975). Henderson *et al.* (1972) were the first to map these rDNA genes on human chromosomes. The satellite region, the nucleolus organizer (NO), on the D and G group chromosomes contains the rDNA genes. Evans *et al.* (1974a) confirmed the placement of the 18 S and 28 S genes on chromosomes 13, 14, 15, 21, and 22. Their data also indicated that not all D and G chromosomes have the same number of rDNA genes. In samples from three individuals, chromosome 14 had the fewest genes, while chromosome 21 seemed to have the most. These chromosomes had been identified by Q-banding using quinacrine hydrochloride, washed in water overnight and hybridized with *Xenopus* ^{3}H-rRNA.

According to Bross and Krone (1972) the human genome has 416 rDNA cistrons per diploid cell or an average of 82 per NO at metaphase. The data of Evans *et al.* (1974a) indicate that the human genome could be polymorphic for the rDNA content for every NO chromosome, some differing by 3 to 4 times the average. The D or G chromosomes with high rDNA values always seem to have a long satellite. Specific 15+ and 22+ variants have been identified. We have similar observations shown in Fig. 4b where a long satellited D group chromosome was annealed with ^{125}I-18 S rRNA. It shows at least twice the radioactivity of other D or G group chromosomes. This chromosome with an extra long satellite and a large "stalk" (Fig. 4a) is probably 13, but it has not been properly identified by Q- or G-banding.

Another individual was examined (Fig. 5) and the data is given in Table 1. On the average the G group chromosomes have nearly twice the amount of label found over D group chromosomes, confirming the observation of Evans *et al.* (1974a). The zero class is more frequent than expected in the D group as

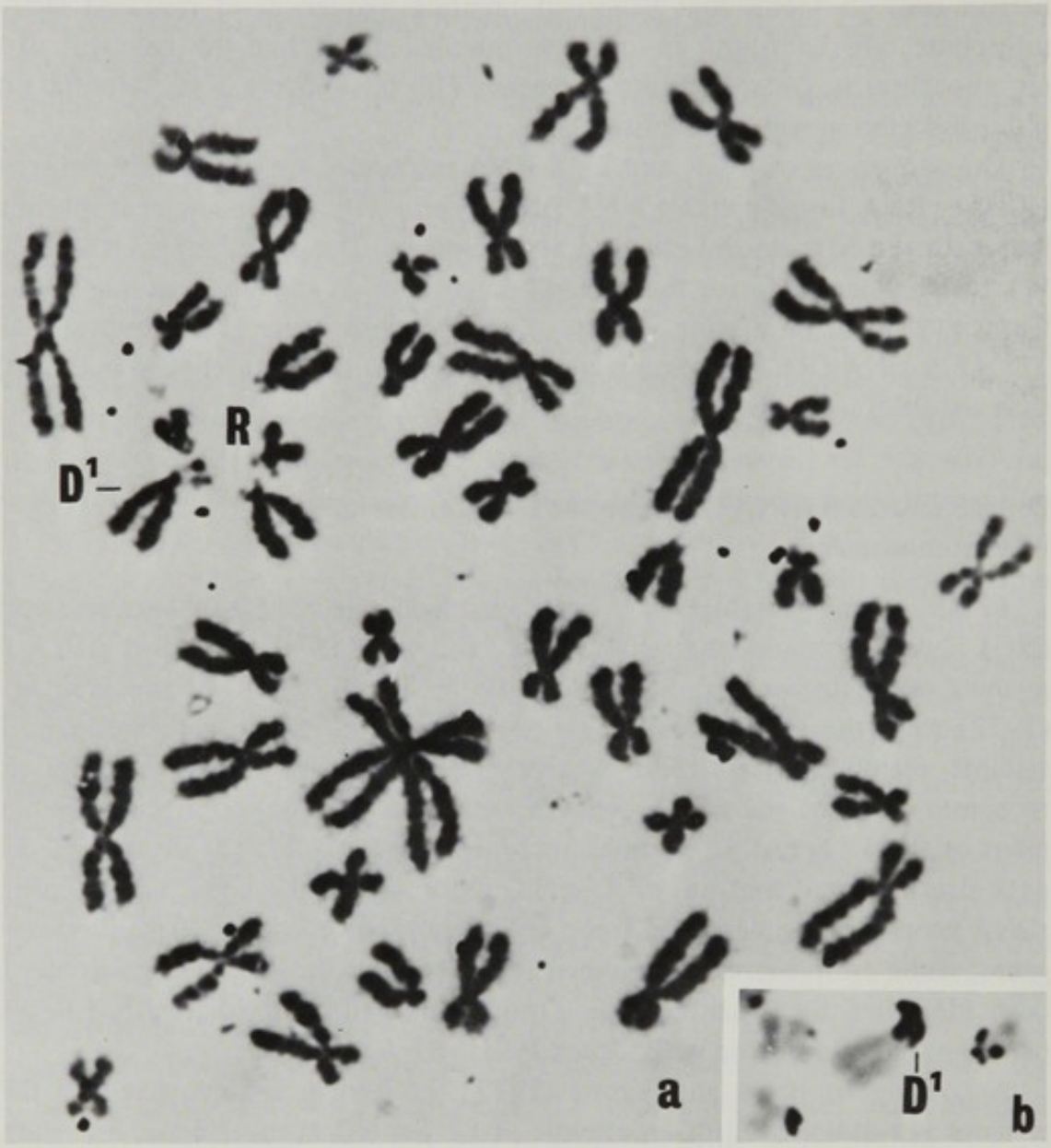

Fig. 4. (a) *In situ* autoradiograph showing a rosette (R) of D and G chromosomes in a remnant association of previous nucleolar activity. One D group chromosome (D^1) has an extra long satellite and large stalk. Chromosomes were annealed with ^{125}I-9 S histone mRNA from HeLa cells (Dr. T. Borun, unpublished). No convincing histone gene site was found. There is a probable contamination from 18 + 28 S rRNA in the mRNA sample. (b) Autoradiograph of D and G group chromosomes using ^{125}I-18 S rRNA. The same D group chromosome (D^1) in (a) shows heavy labeling indicative of 2–3 times the amount of rDNA compared to other D or G group chromosomes.

indicated in Table I. Banding studies will be necessary before the specific chromosome missing the rDNA genes is identified in this normal male (Fig. 5). A comparison of two different individuals identifies a chromosome 15 with high levels of rDNA and a chromosome 22 with low levels (Evans *et al.*, 1974a). The results of Bross *et al.* (1973) from filter hybridization indicate a considerable variation between individuals. Not only are there differences between D and G

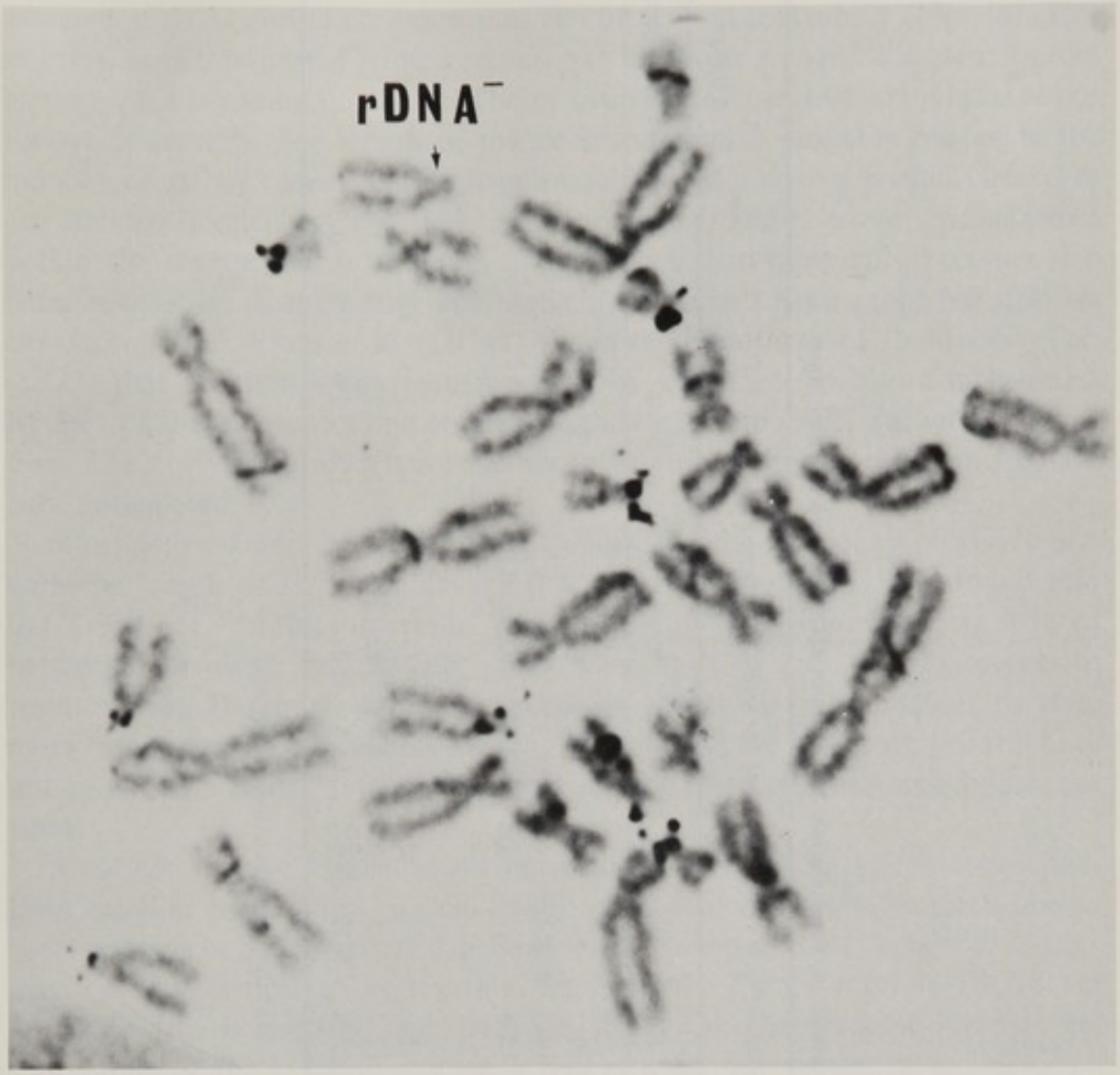

Fig. 5. *In situ* preparation ^{125}I-18 S annealing to D and G group chromosomes of a normal male. A putative rDNA-deficient D group chromosome (rDNA$^-$) is indicated. Quantitative data are given in Table I.

group chromosomes, but dramatic deviations in rDNA content between homologs as indicated by *in situ* data. Detailed family studies should be done, especially where certain D and G group chromosomes can be identified by large stalks or by other cytological markers to determine the inheritance of these rDNA genes. The large range of variation for rDNA content in the human species will require population studies.

C. D and G Group Chromosomes in Rosette Association

The association formed by D and G chromosomes is of considerable interest because these chromosomes are often involved in trisomy and reciprocal translocations, which are genetic phenomena of considerable concern. The rosette

TABLE I
Autoradiographic Analysis of D and G Group Chromosomes for Relative Amounts of rDNA from a Normal Male

	Distribution of silver grain number for each chromosome[a]														Average grain number per chromosome
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	
D group															
No. observed	19	5	10	8	9	4	2	2	1	2					2.5
% observed	30.6	8.1	16.1	12.9	14.5	6.4	4.5	4.5	1.6	3.2					
Expected[b]	8.2	20.5	25.6	21.6	13.3	6.7	2.8	0.9	0.3	0.08					
G group															
No. observed	0	1	7	2	5	3	7	1	7	2	1	1	3	1	5.9
% observed	0	2.4	17.0	4.9	12.2	7.3	17.0	2.4	17.0	4.9	2.4	2.4	7.3	2.4	
Expected[b]	0.3	1.6	4.7	9.4	13.8	16.3	16.0	13.5	10	6.5	3.8	2.0	1.0	0.5	

[a] Data from 11 cells using ^{125}I-18 S rRNA at 1×10^8 dpm/μg annealed *in situ*.
[b] Poisson distribution of the mean in percent.

association of D and G chromosomes can be seen at metaphase (Fig 4a). This association is interpreted to be a remnant of nucleolar activity. Ribosomal genes are sharing a common nucleolus. The *in situ* method can test some ideas about rosette formation. The length of the satellite thread is probably related to the number of rDNA copies and its length could be related to a greater chance of association. Henderson *et al.* (1972) found that D and G group chromosomes within the rosette had little more, if any, rDNA than those chromosomes free from association. Using a large satellited chromosome 22 and a long NO stalk on chromosome 15, Evans *et al.* (1974a) tested the hypothesis of Henderson *et al.* (1973) that a chromosome with more rDNA will often be found in rosettes. Neither of these chromosome segments exhibited preferential nucleolar association. The analysis of association seems to be more complex than might have been anticipated.

Short arm markers have been used to correlate the frequency of association with the length of the nucleolar constriction. Schmid *et al.* (1974) found that the degree of rosette formation, comparing G and D chromosomes, differed between individuals, but usually there were more G group chromosomes in rosettes than D group. Also, chromosomes with long NO constrictions were more often associated than not. These latter data and the *in situ* studies still have not provided a satisfactory explanation for the consistent nonrandom associations.

Although specific "stalks" have been observed, it is not yet known which DNA satellite corresponds to each band, nor have these heterochromatic blocks been mapped in precise detail. The linear arrangement of these molecular species may be very critical to aggregation. Many of the human satellite DNA's are located on the D and G groups. Satellite DNA I is on chromosome 3 and on the short arms of the D chromosomes as well as other chromosomes, including 21 and 22 (Jones *et al.*, 1975). Satellite II is on chromosomes 1, 9, 16, the Y, and probably at sites on the D and G groups. The location of the DNA sequences for satellite III are on chromosomes 9 and the D and G chromosomes. For details see Chapter 9. Specific fluorescent antibodies have been made against 5-methylcytosine and used to bind human chromosomal DNA. The fluorescent sites of 5-methylcytosine were located and correlated with the C bands of satellite II on chromosome 1, 9, 16, Y, and the short arm of 15.

The four main DNA satellites have also been localized by Evans *et al.* (1974b) and by Gosden *et al.* (1975). These authors point out an oversight by previous workers, that the Y chromosome possesses significant amounts of all four DNA satellites. Besides confirming the findings with I and II, satellite DNA III was found primarily on the Y, 9, 15, and 21. There is evidence that alkaline Giemsa staining correlated with satellite DNA III (Bühler *et al.*, 1975). DNA satellite IV had major blocks on the Y and on 13, 14, 15, 21, 22, and 20. Although there is some disagreement about the localization studies above, the general features are

similar. Future mapping studies with the DNA satellites should pay special attention to the linear order of these molecular species on chromosome 9, the Y, and the D and G group chromosomes. Certain translocations, deletions, inversions, and duplications will be useful. Each DNA satellite should be very pure by all available criteria, otherwise the experiment will not be definitive. The quantitative estimates which have been made with *in situ* study may be inaccurate because coincidence was not considered. Here more than one beta track collides with one silver halide crystal. Highly labeled segments will record lower values than they really possess because slides are exposed too long. Many beta particles go undetected because the crystal is already ionized. The best way to attain accurate quantitation is to have four or five different exposure times. The best estimates of the amount of radioactivity will be from the linear part of the curve. This type of analysis has been started for DNA satellites of *D. melanogaster*, starting with IV (1.705) by Peacock and Steffensen (1975).

Before the associations of the nucleolar chromosomes can be understood, we must understand more about the role of the highly repeated sequences in pairing reactions at the nucleolus organizer region. As far as the author is aware, every nucleolus has the rDNA bracketed by heterochromatin. Organisms such as *Drosophila*, maize, mouse, and man have a complex arrangement of heterochromatic blocks on both sides of the rDNA satellite. The reasons for this structural organization are not known, but the organization of the heterochromatin seems to involve ribosome formation.

There are complications and possible artifacts that might be inherent in the analysis of metaphase associations, all too obvious to workers in the field. Most meaningful chromosome affinities are probably disrupted during slide preparation. Even if associations are maintained, *metaphase is not interphase* and important chromosome to chromosome contacts may have disappeared during prophase. This is the reason for a number of recent efforts to examine the interphase nucleus. The ten chromosomes of the D and G groups should be followed through the entire cell cycle "individually," not a simple task to accomplish. Ideally, ten centers of radioactivity should be identified. We do not really know how many of these ten possible rDNA gene blocks are active in making 18 S and 28 S rRNA. Do the ribosome genes and their surrounding heterochromatic DNA have preferential associations that change during the cell cycle? These questions and other unknowns await careful experimentation.

V. CLINICAL APPLICATIONS OF *IN SITU* HYBRIDIZATION

Generally speaking, the technology is now available to map all the RNA molecules associated with protein synthesis. The mapping of the three ribosomal

RNA species 5 S, 18 S, and 28 S rRNA has already been discussed. These were the first to be examined because of the following: (1) the knowledge from filter hybridization showed these genes were highly repeated; (2) calculations predicted that these genes could be mapped using high specific RNA with ^{3}H-RNA or ^{125}I-RNA as a label; (3) ribosomal RNA makes up about 85–90% of the cells so these molecules are relatively easy to isolate and to purify. One need not necessarily use human rRNA, since mouse or *Xenopus* RNA can be used with results little different from those with human RNA.

Only a few clinical studies have been reported. Using blood lympocytes and fibroblasts, Johnson *et al.* (1974) found a small metacentric chromosome had rDNA satellites on both ends. The patient had symptoms similar to the cat-eye syndrome. While mapping the 5 S RNA we (Steffensen *et al.*, 1975) used

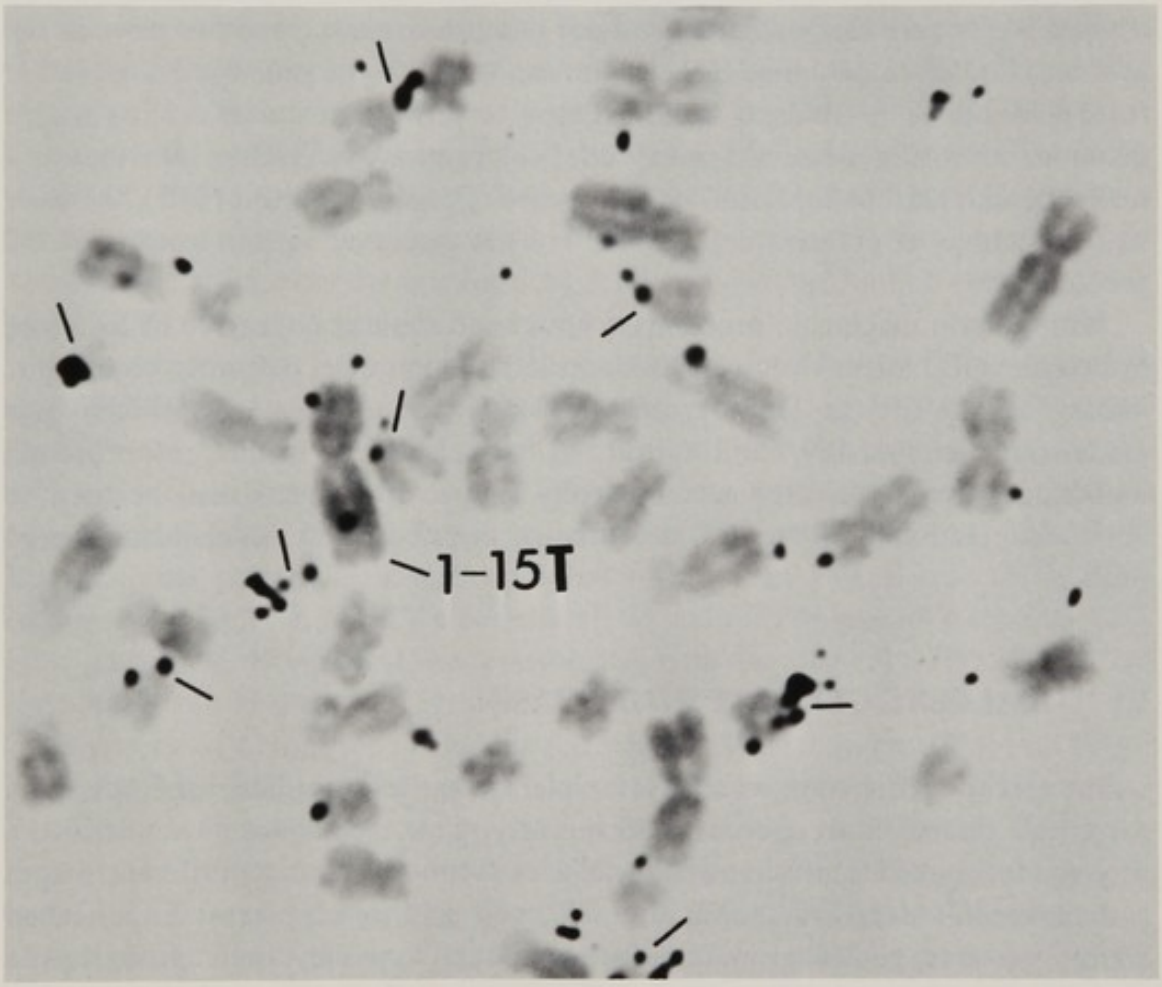

Fig. 6. Autoradiograph of ^{125}I-18 S rRNA hybridized to a metaphase from an individual carrying the translocation t(1;15) (p36;q14), where the centric 15;1 part of the translocation had not been transmitted. This cell and other data confirm that only nine rDNA (NO) regions are present. Labeled NO regions are indicated by a dash (–). The leukocyte preparations were a gift of Dr. Lovrien; cells of this translocation are on file at the Cell Registry in Camden, New Jersey.

translocation, t(1;15) (p36;q14). In one patient the 15;1 part containing the rDNA was missing. These leukocyte slides, a gift of Dr. Lovrien, were hybridized to ^{125}I-18 S rRNA. The loss of the 15 rDNA fragment is confirmed. Only nine rDNA centers were evident in Fig. 6 and in other metaphase figures observed from this individual. This *in situ* evidence provides an obvious confirmation of a missing part of a translocation. The *in situ* method would provide critical proof when an interstitial segment is lost. With short segments G-banding is not always reliable.

With the collaboration of Drs. A. de la Chapelle, M. A. Ferguson-Smith, and P. Szabo (unpublished), some deficiencies on the long arm of chromosome 1 have been examined near the 5 S DNA. One deficiency is probably proximal to the 5 S gene. A second and more distal deficiency may include part of the 5 S genes (P. Szabo and A. de la Chapelle, unpublished). The method can determine if the 5 S genes are really deleted, and not just transposed to another place in the genome. Complete or partial deficiencies can establish the gene for 5 S as well as for 18 S and 28 S RNA. It is not as easy to prove that one is dealing with a deletion, especially when the gene codes for an enzyme. Such a gene may not really be deleted but repressed or transposed. Ferguson-Smith (1975) has dealt with a number of putative deficiencies and has discussed various aspects of the problem.

With certain diagnostic problems, a virus or bacteria may need to be localized in human cells. Adenovirus 12 was identified within nuclei of human embryonic kidney cells (HEK) by Dunn *et al.* (1973). The complementary ^{3}H-RNA was made from the viral DNA and hybridized to nuclei. Some of the centers of viral replication seem to cluster near nucleoli. The *in situ* method can be used to detect infected cells if one has a source of the virus to synthesize the labeled probe.

VI. LOCALIZING GENES AT INTERPHASE

Interphase is the stage when most nuclei are actively synthesizing RNA and a stage least amenable to analysis until recently. New techniques have provided a way to study nuclear differentiation. Nuclei assume all kinds of different shapes and chromatin densities during the cell cycle and development. Experienced histologists and pathologists can distinguish cell types by their characteristic nuclear differentiation. A tactic in present research is to translate biological intuition into physical–chemical reality.

Comings (1968, 1972) reviewed many facets of nuclear organization. He suggested an important role for specific chromosome attachments, particularly those to the nuclear membrane. In some of the first studies with the ultrastructure of *Drosophila* nuclei, Gay (1956) recognized the affinity between hetero-

chromatic segments and the nuclear membrane. Many have yet to appreciate the generalizations recognized by McClintock (1965) in the cytogenetic analysis of the mutable gene systems in *Zea mays*, where she emphasizes that controlling elements are heterochromatic blocks attached to the nuclear membrane. Again we are left knowing the importance of nuclear phenomena in differentiation and genetic regulation but without a mechanism to accomplish the specificity and recognition.

Special staining, fluorescent techniques and *in situ* hybridization have been applied to interphase analysis. Most of the observations are qualitative in nature. A rigorous three-dimensional analysis of nuclei has yet to be done. We have already considered the ribosomal genes, 5 S, 18 S, and 28 S rRNA, and the incomplete information about their positioning at interphase. The nucleolus is the major landmark, so considerable attention should be given to its organization in the nucleus and other complex parameters. Fortunately, polymorphisms involving large C bands on chromosomes 1, 9, and 16 have provided excellent markers. The Y chromosome can be followed by fluorescence using quinacrine hydrochoride or by *in situ* hybridization. Both of the above methods can be used with the same cells. The nucleolus and the nuclear membrane serve as two coordinates. To do a proper analysis to detect asymmetry, one needs at least three markers. Perhaps the late-labeling X chromosomes could be used in female cells. XXY cells would be even more useful.

Most experiments have used the Y chromosome and followed its association with either the nuclear membrane or the nucleolus. Gagné *et al.* (1972) and Wyandt and Iorio (1973) find a high proportion of Y chromosomes associated with nucleoli in lymphocyctes and fibroblasts. In an examination of five cell types Tishler *et al.* (1974) found leukocytes (XYY) with 90–94% of both Y chromosomes peripheral near the nuclear membranes. Buccal cells (XYY) had only 4.7–16.7% with both Y's peripheral, while in fibroblasts (XY) only 6–11% were peripheral. In neurons the Y associates with the nucleolus 98–99% of the time (Iorio and Wyandt, 1973).

A variety of cytogenetic evidence has been examined and analyzed by Vogel and Schroeder (1974) and some of the data will be considered here. Three of the five D and G chromosomes are involved in Robertsonian translocation. The great majority of interchanges are between chromosomes 13 and 14 and between 21 and 14. Other combinations are relatively rare, about ten times below frequency of the combinations above. Preferential translocation must be based on specific associations that are not directly related to rDNA content, since both chromosome 15 and 22 possess high rDNA values but have the lowest chance for translocation. Vogel and Schroeder (1974) present the nonrandom interchange data derived from aberrations produced by Bloom's syndrome, mitomycin C and Fanconi's anemia, all suggesting that the interphase nucleus has internal order.

Future analysis must be directed at the physical and biochemical basis for the

nonrandom association. What is the basis for specific DNA to DNA interactions, DNA to membrane binding and DNA to nuclear pore associations? An important observation has been made with mouse liver nuclear membranes and nuclear pore complexes. Aaronson and Blobel (1974) removed both the outer and inner nuclear membranes using mild detergents. An amorphous layer with the nuclear pores complex remained. At various locations presumptive heterochromatic segments were associated with this outer mass and with the nuclear pores. The chemical nature of this "nuclear cortex" has still to be determined. One would hope that the nuclear pore complex will contain proteins that can recognize specific nucleotide sequences, which in turn hold the chromosome to a particular membrane location (see also Chapter 5).

VII. MESSENGER AND TRANSFER RNA

So far as the author is aware, no one has mapped any gene that codes for a mRNA or tRNA molecule by the *in situ* method on diploid chromosomes, despite some claims to the contrary. We shall consider these claims after looking at the evidence in the Diptera, where salivary chromosomes have a 1000 times more DNA per gene, thus less difficulty in detecting unique sequences or sequences with a few repeats. In *Drosophila* the first published attempt to map tRNA (Steffensen and Wimber, 1971) was a feasibility study, since the tRNA was neither pure nor had high specific activity. Since then, a few tRNA species have been mapped in *Drosophila melanogaster* by Grigliatti *et al.* (1974) and Szabo (1974). Even here the tRNA was not absolutely pure because there were traces of other iso acceptors and/or 5 S RNA. The autoradiographic method can detect contamination that are one and two orders of magnitude below levels of detection of the best techniques in biochemistry. Since it is still difficult to map tRNA genes with salivary chromosomes, mapping with diploid chromosomes is futile.

A number of RNA types have been mapped in *Chironomus* as reviewed recently by Edström and Lambert (1975). The findings are especially interesting where mRNA was isolated from the cytoplasm and then hybridized back to the same cell type and to the puffed gene which was actively making that specific mRNA. In *Drosophila* mRNA was isolated from cells that display heat-induced puffs and annealed back to the same puffs (Lindquist *et al.*, 1975). The gene coding for histone were mapped by Birnsteil *et al.* (1973) to chromosome 2 of *Drosophila melanogaster* at 39CD. The author has confirmed this latter placement with two different samples of histone mRNA, one of which was from HeLa cells. *Drosophila* was used as the control before trying the mRNA on human chromosomes. The histone mRNA from HeLa cells is part of an unpublished and ongoing project of Drs. T. Borun and W. Prensky. So far the human

chromosome localization has shown no sign of specific localization for the histone genes (Fig. 4a). There is an indication that the sample was contaminated with 18 and 28 S RNA fragments. In the sea urchin, the histone genes have been isolated in one block of DNA using restriction enzymes and the plasmid technology by Cohn *et al.* (1975). According to Wilson *et al.* (1974) the human histone genes are repeated 10 to 20 times. We are attempting to map these loci in man using ^{3}H-cRNA made from the sea urchin DNA possessing all five histone genes (Cohn *et al.*, 1975), and these genes may be on a C-group chromosome. Experiments with a few repeated genes are "long shots" and difficult to get a signal above background noise. At least two technical improvements are needed: (1) greater isotope detection, and (2) 100% RNA:DNA hybridization efficiency.

There is a claim by Price *et al.* (1972) and Price *et al.* (1973) that the structural genes coding for hemoglobins have been mapped on human chromosomes using labeled mRNA as well as labeled viral RNA to map complementary sites. These data have been severely criticized for a number of reasons, but mainly because the ^{14}C-RNA used had very low specific activity (Bishop and Jones, 1972; Prensky and Holmquist, 1973). Price and Hirschhorn (1975) have replied to the criticisms. One of their arguments claims that Cot values are meaningless for *in situ* hybridization, which amounts to suspending the laws of thermodynamics. Also these authors misinterpret saturation data derived from filter experiments. The theoretical objections to their globin studies still stand, so their findings remain in question.

Atwood *et al.* (1975a) did control *in situ* experiments with mouse cells where the globin genes had been mapped by other means. Their autoradiographs indicated that the label annealed to the expected mouse chromosomes. Filter disc methods have suggested that the human genes for α- and β-globin are on different chromosomes, according to recent findings (Deisseroth *et al.*, 1976). This controversy will not be settled entirely by *in situ* hybridization but by cell hybridization combined with other techniques. Recent information from the latter authors indicate that the genes for α- and β-hemoglobin are on two different C group chromosomes. With improved *in situ* methods and highly labeled ^{125}I-mRNA for α- and β-globin, we were able to detect label on C group chromosomes but not from the A nor B group.

Another type of *in situ* localization has been done. RNA copies of a gene can assay mRNA in the cytoplasm. Conkie *et al.* (1974) used globin ^{3}H-cRNA made from the globin DNA to monitor the amount of globin mRNA in the cytoplasm in mouse cells transformed by Friend virus and treated with dimethyl sulfoxide. Other work from this group (Harrison *et al.*, 1974) follows the appearance of the globin mRNA in mouse erythroid cells taken from fetal liver. No significant RNA hybridization occurred in other mouse cell types (i.e., lymphoma cells, L cells, or hepatocytes) that are not able to make globin. When the DNA probe is available this technique has a number of uses in localizing and quantitating

mRNA in cells from different tissues. Many aspects of development and gene control can be investigated, especially in tissues where cell types are mixed.

ACKNOWLEDGMENTS

Part of the original research reported was supported by USPHS, grant GM211 23-01.

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4

New Approaches to Human Gene Mapping by Somatic Cell Genetics

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I. INTRODUCTION

The last few years have been very exciting for human geneticists. Great advances have been made in the understanding of the chromosomal or biochemical basis for many genetic diseases. The most explosive development, however, has been in the field of human gene mapping, based largely on the results derived from studies using rodent–human somatic cell hybrids. There has been marked progress in mapping by family studies as well, due in part to stimulation by the somatic cell hybrid results.

Conventional genetic analysis in man has been hampered by his small family size, long generation time, and breeding practices. There has also been a lack of chromosomal and phenotypic markers; gene mapping in man has been based on the determination of autosomal or X-linked inheritance of the genes for a number of inherited syndromes and blood group antigens. There have been two recent waves of advance in human cytogenetics. The first wave followed the discoveries of the use of hypotonic medium (Hsu, 1952) and lymphocyte mitogens (Moorhead *et al.*, 1960; Nowell, 1960). The second wave followed the discovery of various chromosome banding techniques (Caspersson *et al.*, 1970). A great expansion of the battery of human phenotypic markers includes isozymes and other protein polymorphisms detectable by electrophoresis. There has also been a rapid expansion in our understanding of the basic biochemical defects found in many genetic diseases. Many of these new discoveries can be utilized both in family studies and in cell culture, most importantly hybrid cell culture. Thus, it has been the synergistic interaction of somatic cell hybrid and family studies that has resulted in the recent advances.

A number of promising new methods for gene mapping, several of which are extensions of the hybrid cell system, have recently been developed, and will be discussed briefly below; these include Cot analysis of hybrid cells, quantitative mapping using irradiated human cells as parental cells in rodent-human hybrids, gene and chromosome transfer, and ovarian teratoma analysis. *In situ* hybridization, another method with great potential for human gene mapping, is discussed by Steffensen in Chapter 3.

A large number of books and reviews on various aspects of gene mapping and somatic cell hybridization have appeared within the last few years and should be referred to for further discussion and more detailed information. Some books of interest are those by Bergsma (1974, 1975a, 1976), Davidson and de la Cruz (1974), Defendi (1969), Ephrussi (1972), Harry Harris (1975), Henry Harris (1970), Morgan Harris (1964), Hood *et al.* (1975), McKusick (1975), Pollack (1975), Puck (1972), and Race and Sanger (1975). Reviews of interest include those on somatic cell hybridization and gene mapping by Davidson (1970), Giles and Ruddle (1973b), Gordon (1975), Grzeschik (1973), Migeon and Childs (1970), Nichols and Ruddle (1973), Ruddle (1972, 1973, 1975), Ruddle and

Creagan (1975), Ruddle and Kucherlapati (1974), Sell and Krooth (1972), and Zeuthen (1975); on the use of somatic cell hybridization for analysis of differentiated functions by Bernhard (1976), Darlington and Ruddle (1975), Davidson (1974), Davis and Adelberg (1973), and Gordon (1975); on human gene mapping studies by McKusick and Chase (1973), Renwick (1969, 1971), Robson (1972), Sanger and Race (1970), and Weitkamp (1972); on mutation and selective systems by Chu (1974); Clements (1975), DeMars (1974), Kao and Puck (1974, 1975), Siminovitch (1976), and Thompson and Baker (1973); and on *in situ* hybridization by Price and Hirschhorn (1975) and Wimber and Steffenson (1973).

II. FAMILY STUDIES

We will review very briefly the methods used and some exemplary results from family studies. More comprehensive recent reviews are those by McKusick and Chase (1973), Race and Sanger (1975), Renwick (1969, 1971), Robson (1972), and Weitkamp (1972); also see McKusick (1975).

A. X-Linkage versus Autosomal Linkage

Family studies can readily determine whether the gene for a phenotype can be assigned to either the X chromosome or to an unspecified autosome. The hallmark of X-linkage is absence of male-to-male transmission. The alternative of sex-limited expression has to be considered in such conditions as the testicular feminization syndrome (lack of androgen receptors), since males with this condition are infertile and can not reproduce. In this instance, by studying the cells of a heterozygous mother of an affected male, Meyer *et al.* (1975) could demonstrate populations of cells with and without androgen receptors, thus providing strong evidence for the X-linkage of this trait. The relative ease of establishing X-linkage is demonstrated by the fact that the first assignment to the X chromosome was that of a gene for color blindness in 1911 (Wilson, 1911). The first assignment to a human autosome was not made until 1968 (Donahue *et al.*, 1968).

B. Gene–Gene Linkage

Studying families with appropriate patterns of polymorphisms at different loci allows the determination of intergene distance as measured by frequency of meiotic recombination. Obviously, loci with a large number of alleles and those with a high incidence of at least two alleles are most useful for family studies. Family studies are uniquely useful for establishing the assignment of loci for

genetic diseases which may be recognizable, at least initially, only at the organismal level. The ability to quantify intergene distance is also an advantage of family studies over somatic cell hybrid studies, although the possibility of quantification using somatic cell hybridization now exists (Goss and Harris, 1975) (see Section V,A).

Genes can be indirectly assigned to chromosomes if they can be shown to be linked to a gene that is already assigned to a specific chromosome. For example, the gene for LCAT (lecithin:cholesterol acyltransferase) has been assigned to chromosome 16 by virtue of linkage to the α-haptoglobin gene (Teisberg and Gjone, 1974), which was previously assigned to chromosome 16 (Robson *et al.*, 1969).

C. Gene–Chromosome Linkage

Inherited polymorphisms of chromosome structure or staining characteristics (presumably reflecting underlying differences in DNA sequences) can be used to assign polymorphic loci directly to chromosomes. This is done by studying the joint pattern of inheritance of the chromosomal and other genetic markers. Nonindependent assortment indicates that the chromosome marker is linked to the locus in question, with the recombination fraction giving a measure of the distance. The first assignment of a human gene to a specific autosome, the assignment of the Duffy blood group locus to chromosome 1 (Donahue *et al.*, 1968), was made by showing linkage of the Duffy locus to an inherited polymorphic increase in the centric heterochromatin on chromosome 1 (then thought to be an inherited "uncoiled" region). The first localization of a locus to a specific chromosome arm, the assignment of the α-haptoglobin locus to 16q, was made in a similar fashion (Hecht *et al.*, 1971, Magenis *et al.*, 1970; Robson *et al.*, 1969).

D. Gene Dosage

We have included this section on gene dosage under family studies, since much of the work in man to date has involved material derived from patients with chromosomal abnormalities. The extensive treatment of this subject reflects our belief that gene dosage represents an untapped resource of great potential in gene mapping and gene regulation studies.

The use of gene dosage effects for human gene mapping has been of interest since the discovery of dosage compensation by X-inactivation (the Lyon hypothesis) and the chromosomal basis of Down's syndrome (trisomy 21). Studies attempting to use gene dosage for mapping purposes can be classified as qualitative or quantitative. We can define qualitative gene dosage studies as those which compare the number of different allelic gene products expressed to the number

of genes present. Quantitative studies attempt to correlate the number of genes present with changes in the *quantity* of gene expression, such as the amount of a specific protein or enzyme activity.

1. Qualitative Dosage

The use of qualitative gene dosage for mapping is based largely on the hypothesis that concurrent rare genetic events are likely to be related. Thus, if we find only one allele at a gene locus in an individual that is expected to have two different alleles, and concurrently we find a chromosome deletion in that individual, a possible explanation is that the deleted chromosome segment contains the locus in question. Alternative explanations would include a null-allele at the locus not causally related to the deletion. That this latter possibility must be seriously considered was shown by Cook *et al.* (1969) who demonstrated that in one such case the apparent loss of an allele at the α-haptoglobin locus concurrent with the finding of an aberrant chromosome 13 was due to a null-allele at the α-Hp locus (also see Robson, 1972). The α–Hp locus is actually on chromosome 16 (Robson *et al.*, 1969). A possible assignment using this method is that of the Kidd (*Jk*) locus to chromosome 7 (Shokeir *et al.*, 1973).

Another qualitative method would be the correlation of expression of three different alleles at a single locus concurrent with the finding of trisomy of a chromosome or chromosome segment. There are not any likely alternative mechanisms for this finding, but the potential usefulness of this method is limited, since few loci demonstrate the degree of polymorphism necessary for such a genetic event to be likely. This method has not proved useful to date. A population genetics approach comparing allele frequencies in disomic and trisomic states, the genotype-frequencies method of Bateman (1960), is unlikely to be useful except possibly in trisomy 21 (Renwick, 1969).

One can look at qualitative dosage from another angle. If one finds heterozygosity at a locus, concurrent with a clear deletion or monosomy, the heterozygous locus can be *excluded* from the missing region of the genome. This *exclusion mapping* could be very helpful in determining where *not* to look for gene assignment or linkage and could eventually determine the correct location of a gene by ruling out all possible alternatives. While early attempts were made to collect this data systematically (Bender and Burchkardt, 1970), the advent of the chromosome banding methods make even more worthwhile a concerted effort to collect this kind of data (Ferguson-Smith, 1975).

A more specialized type of gene dosage has been useful in mapping the X chromosome. Females who are genetically heterozygous at an X-linked locus will have only one or the other of their X chromosomes active in any one cell or its clonally derived progeny. Thus, the gene for testicular feminization (dihydrotestosterone receptor gene) was shown to be an X-linked, as opposed to a sex-limited autosomal dominant gene, by the demonstration of two populations

of cells, one with and the other without the dihydrotestosterone receptors, in the heterozygous mother of an affected male (Meyer *et al.*, 1975).

2. Quantitative Dosage

The assumption underlying quantitative dosage studies is that the quantity of gene product found is proportionally related to the number of copies of the gene present. This assumes that there is no feedback of the amount of a gene product on transcription or translation, and that the processes of transcription, translation, modification, and degradation do not involve steps strongly rate-limited by factors apart from the number of genes and amount of gene product. The assumption that given an otherwise constant or almost constant background genome, the x number of copies of a gene will give x/y ($y \neq 0$) times as much product as y copies has been shown to be a valid approximation for a number of eukaryotic systems besides man including yeast (Nelson and Douglas, 1963), barley (Nielsen and Frydenberg, 1971), wheat (Brewer *et al.*, 1969), *Datura* (Carlson, 1972), *Drosophilia* (Grell, 1962; Lindsley *et al.*, 1972; Stewart and Merriam, 1974), and mouse oocytes (Epstein, 1969, 1972; Kozak *et al.*, 1974). Increases in the amount of a gene product per cell proportional to increases in ploidy (additional *complete* chromosome sets) have been shown for glucose-6-phosphate dehydrogenase (G6PD) in triploid rabbit embryos (Bomsel-Helmreich, 1970), for collagen in rat fibroblasts (Priest and Priest, 1969), and for three enzymes in Chinese hamster cell lines (Westerveld *et al.*, 1972). Relatively quantitative gene dosage effects were shown in heteroploid mouse cell lines by examination of isozyme patterns (Ruddle, 1968; Farber, 1973) and correlation with chromosome variation (Farber, 1973).

Based on the wide variety of studies cited above, it would be expected that quantitative gene dosage would be useful for gene mapping in man. In fact, a number of attempts were made to assign genes to chromosome 21 based on elevated levels in individuals with trisomy 21. Many of the earlier suggested assignments using material with trisomy 21 as well as with other chromosome abnormalities have been shown to be questionable (Pantelakis *et al.*, 1970; McKusick and Chase, 1973; Renwick, 1969, 1971; Robson, 1972) and a cautious attitude has developed about studies utilizing gene dosage effects for mapping purposes.

Support for gene product proportionality to gene number in man has come from a related area. For a number of inherited diseases caused by homozygosity for alleles coding for enzymes with low or no activity, heterozygotes have enzyme activities intermediate between normal homozygotes and affected homozygotes. For mutant alleles with no activity, heterozygotes in most cases demonstrated approximately 50% of the level of activity found in homozygous normals, and could thus be detected (cf. Harris, 1975; Raivio and Seegmiller, 1972).

More recent studies have attempted to demonstrate dosage effects for human

gene products previously assigned to specific human chromosomes by other methods. For example, increased levels of acid phosphatase 2 in an individual trisomic for part of chromosome 2 (Magenis *et al.*, 1975) supported the earlier assignment to chromosome 2 by qualitative dosage (Ferguson-Smith *et al.*, 1973) and by somatic cell hybridization (Povey *et al.*, 1974). The gene for galactose-1-phosphate uridyltransferase has been conflictingly assigned to either chromosome 2 (Sun *et al.*, 1974) or to chromsome 3 (Tedesco *et al.*, 1974). The assignment to chromosome 3 has been supported by the demonstration of increased levels in cells from an individual with partial trisomy of chromosome 3 (Allderdice and Tedesco, 1975). Increased levels of glutathione reductase in trisomy 8 (de la Chapelle *et al.*, 1971) supported the later independent assignment to chromosome 8 using somatic cell hybrids (Kucherlapati *et al.*, 1974). Mayeda *et al.* (1974) showed decreased levels of lactate dehydrogenase B in an individual monosomic for the short arm of chromosome 12, thus supporting the prior assignment to chromosome 12 (Chen *et al.*, 1973) and localizing the gene to the short arm of chromosome 12. The localization to the short arm of chromosome 12 was independently shown by somatic cell hybrid studies (Jongsma *et al.*, 1975). The level of adenine phosphoribosyltransferase is proportionately raised in trisomy 16 (Marimo and Gianelli, 1975).

Several studies have shown increased levels of cytoplasmic superoxide dismutase in trisomy 21 and suggested localization of the gene to certain sub-bands of chromosome 21 (Sinet *et al.*, 1974; Sichitiu *et al.*, 1974; Crosti *et al.*, 1976). The dosage effect of the antiviral-sensitivity gene (probably an interferon receptor gene), which has also been assigned to chromosome 21 (Tan *et al.*, 1973) has been examined in cells from individuals monosomic, disomic (normal), and trisomic for chromosome 21 (Tan *et al.*, 1974). Instead of a linear relationship, a logarithmic increase in sensitivity with gene dosage was seen (Tan, 1975). This interesting finding is difficult to interpret at present, as the nature of the actual gene product is not known.

Gene assignments in man using quantitative dosage, which have not yet been confirmed, are those for phosphofructokinase to chromosome 21 in trisomy 21 (Pantelakis *et al.*, 1970), and Hageman factor to chromosome 7q based on half-normal levels of activity in two unrelated individuals with deletions of chromosome 7 (de Grouchy and Turleau, 1974). α-Thalassemia has been shown to be due to a deletion of genes for α-hemoglobin chain but no chromosome assignment was made since the deletion was probably undetectable using current cytogenetic techniques (Ottolenghi *et al.*, 1974; Taylor *et al.*, 1974).

3. Possible Future Uses of Gene Dosage Effects in Human Gene Mapping

The current use of gene dosage effects for human gene mapping has been to confirm assignments to chromosomes, and to suggest localization of genes to subchromosomal regions. The regional localization of genes previously assigned

to a particular chromosome should be readily accomplished by examining dosage effects in cells from individuals monosomic or trisomic for overlapping regions of the particular chromosome. A concerted effort to collect and make available such lines should be rewarding. The extensive collection of Y-autosome translocations generated in *Drosophila* (Lindsley *et al.*, 1972) is a model for such a collection of human cells. The use of such panels of cells with partial deletions and additions could well prove more efficient than somatic cell hybrid studies using human parental cells with translocations (see Section III,G,6). Such panels could also be highly useful for subchromosomal gene localization using Cot analysis (see Section V,C), particularly since the actual DNA of the genes should show strict dosage.

As our understanding of and confidence in gene dosage mapping increases, the use of cells with complete and partial monosomies and trisomies for *initial* assignments should increase. This type of mapping could prove very useful in assigning genes found only in differentiated cell types, providing the differentiated cell cultures are available (a potential source of differentiated cell cultures with defined chromosome abnormalities is abortus material). Results from the *Drosophila* Y-autosome system suggest that this type of system may also be useful for studying eukaryotic gene regulation (Rawls and Lucchese, 1974; see also Carlson, 1972).

Finally, a proportional increase in the amount of cell surface antigens with gene dose has been demonstrated (Ostrand-Rosenberg, 1975). By the use of the fluorescent-activated cell sorter (Hulett *et al.*, 1973; Kreth and Herzenberg, 1974) and the appropriate chromosome-specific antisera, it may well be possible to select populations of human or hybrid cells with different numbers of copies of particular human chromosomes. Apart from the use of such lines for gene mapping, these could provide cell lines which produce specific products at high levels.

E. Linkage as Indicated by Protein Structure

Close linkage of genes can be inferred by detection of proteins which presumably arose from combinations of two related genes. Thus, the close linkage of the genes for the hemoglobin β chain and hemoglobin δ chain was suggested by the finding of Lepore-type hemoglobins which have amino acid sequences indicating recombination of the genes for the β and δ chains (Huehns and Shooter, 1965; Giblett, 1969). The finding of "anti-Lepore" recombination products has strengthened the suggested β–δ linkage (Lehmann and Charlesworth, 1970; Ohta *et al.*, 1971). Hemoglobin Kenya, an apparent combination of the δ and γ chains indicates that the β, δ, and γ chain genes are probably closely linked or contiguous (Kendall *et al.*, 1973). The finding of a Lepore-type immunoglobulin heavy chain suggests that there is close linkage of the genes for

the heavy chains of immunoglobulins as well (Kunkel *et al.*, 1969; Van Loghem, 1971).

III. SOMATIC CELL HYBRIDIZATION

A. History

The first clear example of proliferating cells arising from mammalian cell fusion *in vitro* was observed by Barski *et al.* (1960, 1961) in the course of an attempt at cell transformation by co-cultivation of two related heteroploid mouse cell lines. By chromosome analysis Barski showed that a new cell type had arisen containing the chromosomes of both parental lines. Sorieul and Ephrussi (1961) quickly confirmed Barski's results and extended them to other intraspecific combinations including the first hybrids between diploid mouse cells and heteroploid cell lines (Scaletta and Ephrussi, 1965).

It should be noted that all of these early hybrids could be isolated because of inherent growth advantages of the hybrids over the parental cells, though this advantage was sometimes seen only at lower temperatures (Ephrussi, 1965). Not all hybrid combinations, however, displayed this "hybrid vigor" phenomenon and since, in addition, the frequency of hybridization was often quite low, other selective systems were sought. This was accomplished when Littlefield (1964) demonstrated that the HAT (hypoxanthine-aminopterin-thymidine) system could be used to isolate hybrids between two drug-resistant cell lines (see Section III,F). A half-selection modification of the HAT system, which required only one parental line to be drug-resistant, was soon developed (Davidson and Ephrussi, 1965).

Even with clean selective systems, the low spontaneous fusion rate was a problem. This was circumvented by the discovery by Harris and Watkins (1965) that Sendai virus could induce the formation of interspecific heterokaryons. It had earlier been shown that fusion of Ehrlich ascites cells resulted from virus treatment (Okada, 1958, 1962) but the potential for heterokaryon or hybrid cell formation had not been exploited. While Ephrussi and Weiss (1965) first showed that proliferating interspecific (rat–mouse) hybrids could be isolated, Yerganian and Nell (1966) produced the first proliferating hybrids using the virus-fusion technique.

A very important next step, particularly from the human gene mapping viewpoint, was the isolation of the first proliferating human–rodent hybrids by Weiss and Green (1967). The fortuitous preferential and rapid loss of human chromosomes from these hybrids provided the key to their use for human gene mapping. Since Weiss and Green had used a thymidine kinase-deficient mouse cell line, the mouse–human hybrids selected in HAT medium were required to

retain the human gene for thymidine kinase (TK) and hence the human chromosome on which the gene for TK was located. After a number of generations in culture, the majority of hybrid cells had apparently retained only one chromosome in common. It was first thought to be a C group chromosome (Weiss and Green, 1967) but subsequent studies demonstrated that the selected chromosome was of the E group (Migeon and Miller, 1968).

Despite the difficulty in identifying specific chromosomes in the hybrid cells it was apparent that, in the absence of chromosome breakage, phenotypes coded by genes on a single chromosome would be retained and lost as an intact group. Thus, in a large number of hybrid lines (independently derived to rule out the presence in common of an undetected translocation), the coretention and loss of phenotypes was taken as presumptive evidence for assignment to the same (unidentified) chromosome. The first human genes determined to be syntenic* using somatic cell hybrids, based on concordant retention and loss, were the genes for hypoxanthine phosphoribosyltransferase (HPRT) and G6PD (Nabholz *et al.*, 1969). The demonstration in hybrid cells of synteny for these genes was consistent with the prior assignment of these genes to the X chromosome through family studies. Demonstration of this synteny group was facilitated because the HPRT phenotype could be selected for or against using respectively, HAT or 8-azaguanine. The first autosomal synteny demonstrated, using hybrids, was that between the genes for lactate dehydrogenase B and peptidase B (Ruddle *et al.*, 1970; Santachiara *et al.*, 1970).

The problem of unreliable chromosome identification was the last major problem for hybrid cell utilization for mapping. Ruddle and co-workers attempted to identify the specific human chromosomes by computer analysis of orcein-stained hybrid cells (Ledley and Ruddle, 1966), but the breakthrough came with the discovery by Caspersson *et al.* (1970) that quinacrine mustard and similar fluorescent dyes revealed a reproducible banding pattern unique for each chromosome. Modifications of this method utilizing nonfluorescent dyes such as Giemsa (Sumner *et al.*, 1971; Schnedl, 1974), along with quinacrine staining revolutionized the field of cytogenetics, and finally permitted the reliable identification of human chromosomes in the hybrid cells. The first use of this

*The term *linkage* was originally used to describe nonrandom assortment of genes at meiosis, but was later also applied to assignment of genes to sex chromosomes (X-linkage) or to autosomes (autosomal linkage). Renwick (1969) suggested the use of the term *syntenic* to describe the relationship between two genes which could be shown to be on the same chromosome; these genes would not necessarily show linkage in terms of meiotic recombination. The distinction is more important in the negative, i.e. genes that can be shown *not* to be linked may well be syntenic. While the terms X-linkage and autosomal linkage are embedded in common usage, the use of the word *assignment* is preferred to indicate the location of a gene on a specific autosome. Thus *TK* is *assigned* to human chromosome 17, not *linked* to chromosome 17.

TABLE I
Milestones in Somatic Cell Hybridization from the Human Gene Mapping Viewpoint (1960–1975)

1960	Discovery of *in vitro* somatic cell hybridization (Barski *et al.*, 1960)
1964	Biochemical selection of hybrid cells (HAT selection) (Littlefield, 1964)
1965	Half-selection technique (Davidson and Ephrussi, 1965)
1965	Use of virus-induced fusion to form homokaryons and heterokaryons (Harris and Watkins, 1965)
1965	First proliferating interspecific hybrids (rat–mouse) (Ephrussi and Weiss, 1965)
1966	First proliferating hybrids produced using Sendai virus (Yerganian and Nell, 1966)
1967	First human–rodent hybrids–demonstration of preferential loss of human chromosomes (Weiss and Green, 1967)
1969	First hybrid cell confirmation of gene assignment and synteny (*G6PD* and *HPRT* to X chromosome) (Nabholz *et al.*, 1969)
1970	First autosomal syntenic group established using hybrids (*LDH-B*/PEP B) (Ruddle *et al.*, 1970; Santachiara *et al.*, 1970)
1970	Quinacrine mustard fluorescence banding discovered (Caspersson *et al.*, 1970)
1971	First autosomal assignment of a selectable gene (*TK* to 17) (Miller *et al.*, 1971)
1972	First autosomal assignment of nonselectable gene using hybrids (*LDH-A* to 11) (Boone *et al.*, 1972)
1972	First regional localization using hybrids (*TK* to 17q) (Boone *et al.*, 1972)
1972	First use of nutritional auxotrophs for human gene mapping (Jones *et al.*, 1972)
1973	First regional localization using human parental translocation (*HPRT, PGK, G6PD*, to Xq) (Ricciuti and Ruddle, 1973a)
1973	First gene transfer using purified metaphase chromosomes (McBride and Ozer, 1973)
1975	Clone panel method proposed (Creagan and Ruddle, 1975)
1975	Use of irradiated human parental cells for quantitative mapping in hybrids demonstrated (Goss and Harris, 1975)

method was to confirm that the chromosome carrying the TK gene was indeed an E group chromosome, in fact, chromosome 17 (Miller *et al.*, 1971; Boone *et al.*, 1972).

The ready identification of chromosomes made it unnecessary to have to rely on selective retention of a chromosome for gene assignment. All human chromosomes could be identified in the hybrid cells and their presence or absence correlated with the presence or absence of specific human phenotypes such as isozymes. Using this correlation technique, Boone *et al.* (1972) assigned the gene for human lactate dehydrogenase A to chromosome 11.

The first regional assignment (assignment to a subchromosomal region) using hybrid cells, was the assignment of TK to the long arm of chromosome 17 (i.e., 17q) using a hybrid line in which the long arm of 17 had been translocated to a mouse chromosome (Boone *et al.*, 1972). A more systematic attempt at regional localization came with the use of rodent-human hybrids made with human

parental cell lines carrying translocations (Grzeschik *et al.*, 1972). Using a human parental line carrying an X/14 translocation, Ricciuti and Ruddle (1973a,b) assigned the genes for G6PD, PGK, and HPRT to the long arm of the X chromosome. These results have been confirmed and this method and modifications discussed below have been used for the regional assignment of a number of genes (Bergsma, 1974, 1975a, 1976).

As the methods of characterizing hybrid clones were refined, it became clear that use of a small number of well-characterized hybrid clones could lead to more efficient mapping and thereby facilitate the use of phenotype assays that were difficult or expensive. Construction and use of such a clone panel (Creagan and Ruddle, 1975), which theoretically could be as small as five hybrid lines, will be discussed below.

A recent development, the use of rodent-human hybrids made with irradiated human parental cells for quantitative gene mapping, may allow ready establishment of gene order and intergene distances (Goss and Harris, 1975; Goss, 1976). Two further hybrid cell methods which should prove to be powerful tools for fine genetic mapping are gene or chromosome transfer (McBride and Ozer, 1973; Ruddle and McBride, 1976), and Cot analysis of hybrid cells (Deisseroth, 1976; Deisseroth *et al.*, 1976).

B. Cell Fusion

The fusion of somatic cells *in vivo* is an unusual and closely controlled process, as in the fusion of myoblasts to form multinucleated myotubes. It had been observed *in vitro*, however, that viruses could induce multinucleated cell formation, and this phenomenon had been closely examined by Okada (1958, 1962). Harris and Watkins (1965) first clearly demonstrated the usefulness of virus-induced fusion for the formation of intraspecific and interspecific heterokaryons, and in fact true hybrids with one nucleus containing genetic material from both parental nuclei. However, the first proliferating hybrids produced via virus-induced fusion were isolated by Yerganian and Nell (1966).

Sendai virus, inactivated either by UV light or β-propiolactone, has been the most widely used fusion agent, and methods for its production and use have been reviewed (Croce *et al.*, 1972; Giles and Ruddle, 1973a,b; Gordon, 1975; Klebe *et al.*, 1970; Poste, 1972; Stadler and Adelberg, 1972).

There have been objections to the use of a virus, even if inactivated, for cell fusion, and nontoxic, nonviral fusion-inducing chemicals have been sought. Also, some cells (e.g., *Drosophila* cells) cannot be effectively fused with Sendai virus due to the lack of surface viral receptors (Bernhard, 1976). Lysolecithin has been used with some success, but its toxicity has limited its use (Ahkong *et al.*, 1972; Croce *et al.*, 1972; Poole *et al.*, 1970). Glycerol monooleate has been shown to be useful for fusion and hybrid cell production (Ahkong *et al.*, 1973;

Cramp and Lucy, 1974). Liposomes (unilamellar vesicles) prepared from a number of phospholipids have also been reported to be useful fusion agents (Papahadjopoulos *et al.*, 1973, 1974). Pontecorvo (1975) and others (Davidson and Gerald, 1976; Davidson *et al.*, 1976) have shown that polyethylene glycol (PEG) which had first been used to fuse plant cells (Kao and Michayluk, 1974) could very effectively induce hybrid formation in mammalian cells. PEG has been shown to be useful for fusion of *Drosophila* cells in culture by Bernhard (1976). The advantages of PEG in sperm-somatic cell fusion have been demonstrated by Elsevier and Ruddle (1976). A modification of the PEG method using DMSO in addition to PEG has been reported (Norwood *et al.*, 1976).

Surgical fusion of cells as demonstrated by Diacumakos and Tatum (1972; Diacumakos, 1973) is a useful method for fusion of specific cells and isolation of hybrids without selection. Surgical fusion also avoids possible viral or chemical modifications, but this system has not been extensively utilized, perhaps due to the need for considerable technical expertise.

C. Chromosome Identification

Gene mapping relies heavily on staining methods which make possible reliable identification of intact human chromosomes, and allow detection and identification of chromosomal rearrangements such as deletions and intra- and interspecific translocations. Earlier attempts to identify chromosomes based on lengths and arm ratios were not very discriminating, and the use of specific replication patterns detected by autoradiography of ^{3}H-thymidine-labeled chromosomes was very cumbersome.

The breakthrough came with the discovery by Caspersson and co-workers (1970) that fluorescent dyes such as quinacrine mustard could reveal reproducible banding patterns unique for each chromosome. The quinacrine banding was quickly followed by a number of staining methods which when used in various combinations can very accurately identify chromosomes or chromosome regions. The various banding methods include Giemsa banding (Sumner *et al.*, 1971; Seabright, 1972; Wang and Federoff, 1972), constitutive heterochromatin banding (Arrighi and Hsu, 1971; Chen and Ruddle, 1971; Yunis *et al.*, 1971) reverse-banding (Dutrillaux and Lejeune, 1971), Hoechst 33258-staining (Hilwig and Gropp, 1972; Kucherlapati *et al.*, 1975; Lin *et al.*, 1974), Giemsa-11 banding (Bobrow *et al.*, 1972; Bobrow and Cross, 1974; Friend *et al.*, 1976a,b; Gagne and Laberge, 1972), NOR-staining (Goodpasture and Bloom, 1975), and staining with fluorescence-labeled antibodies against DNA (O. J. Miller *et al.*, 1974). A bibliography of earlier references to banding methods has been published (Nilsson, 1973; also see Schnedl, 1974). Many "recipe" articles concerning the art and magic of chromosome banding appear in the *Mammalian Chromosomes Newsletter*. A recommended code for describing banding techniques has

been published (Bergsma, 1975b). *In situ* annealing (Pardue and Gall, 1970) with various satellite or gene specific probes such as cDNA for the ribosomal RNA genes (Henderson *et al.*, 1972) or cDNA specific for individual chromosomes (Kunkel *et al.*, 1976) can reinforce the staining methods.

Of particular value in the analysis of hybrid cells is the use of complementing combinations of methods. One triad useful in mouse-human hybrids is either Giemsa or quinacrine banding which reveal similar banding patterns, Hoechst 33258 which selectively stains mouse centromeric satellite regions very brightly, and Giemsa-11 which stains mouse chromosomes magenta and human chromosomes blue. The remarkable staining properties revealed by Giemsa-11 staining can actually be used to identify rodent–human translocations as the rodent and human components of the translocation chromosomes retain their species-specific staining properties (Friend *et al.*, 1976a,b). Sequential staining of a single metaphase, such as with quinacrine followed by Giemsa-11, is possible (see Chapter 7).

D. Genetic Markers

The term genetic marker originally referred to properties which were observed to be qualitatively inherited (e.g., red flowers in the progeny of plants with red flowers). With the recognition of the Mendelian pattern of inheritance and the role of the chromosomes, the distinction between the observed phenotypic character and the inherited gene became clearer. Subsequent genetic studies refined the gene concept, culminating in the discovery of the structure of DNA and of the actual gene, and the code and mechanisms used to transcribe and translate the information carried by the gene. Thus the connection was made between the ephemeral gene and the genetic markers by which the genes had been detected. While genetic markers are certainly still essential as indicators of the presence of associated genes, we are now able, in a few cases, to detect with probes such as labeled cDNA, the base sequences that make up the actual genes themselves (Price and Hirschhorn, 1975; Wimber and Steffensen, 1973). In fact, our level of detection of genes can be at any of a number of levels removed from the DNA base sequence, or at the level of the base sequence itself. A further consideration is that the inheritance and localization of specific reiterated base sequences has been demonstrated (Rae, 1972). While these sequences are probably not transcribed and do not fit the usual definition of genes they could certainly be considered genetic markers. Thus genetic markers can perhaps be best defined as specific inherited DNA sequences or products which are derived from and reflect such sequences.

For purposes of discussion we can arbitrarily establish a number of levels at which genetic marker analysis is possible.

1. DNA base sequence (e.g., actual genes or other inherited DNA base sequences).

2. Transcribed products (e.g., heterogenous nuclear RNA, tRNA, 45 S ribosomal RNA).

3. Processed transcribed products (e.g., mRNA, modified tRNA, 18 S and 28 S rRNA).

4. Translated products (e.g., enzymes, proenzymes, structural proteins).

5. Modified translated products (e.g., activated enzymes, modified structural proteins, glycoproteins).

6. Specific products of 4 and 5 (e.g., metabolic products).

7. Events or complex phenotypes which can be related to genetic elements (e.g., higher order structures, regulatory phenonema, disease states).

The range of genetic markers will of course vary with the system under study. We cannot, for example, measure intelligence (whatever current definition is accepted) in cells in culture. In human genetics the principal markers used have been human disease syndromes, blood group antigens and, more recently, electrophoretic mobility variants of serum proteins, enzymes, and other proteins. The most common markers examined in cells in culture are translated products or modified translated products. For example, the electrophoretic mobility differences observed between most mouse and human isozymes have made these the principal genetic markers used in somatic cell hybrid mapping studies. In fact, the goal of assigning the gene for at least one easily tested isozyme to each human chromosome has almost been achieved (see Section IV).

The use of probes for genetic markers at levels 1, 2, and 3 (see Section V,C) should increase greatly as our ability to isolate and purify the appropriate probes increases. The advent of cloning of eukaryotic DNA via bacterial plasmids should greatly enhance the possibilities here (Williamson, 1976).

The use of facultatively expressed genetic markers including differentiated cell markers such as albumin production, is an area of active progress. Of great importance here is the demonstration of human albumin production in hybrids between mouse hepatoma (albumin producers) and human leukocytes (not producing albumin) (Darlington *et al.*, 1974).

Finally it should be noted that many of the genetic marker systems have been derived from, or can potentially be applied to, studies of fetal cells grown from amniotic fluid or isolated from maternal blood, and may thus be useful in prenatal diagnosis and monitoring.

E. Chromosome Loss

The formation of rodent–human hybrid cells was not by itself useful for human gene mapping. What was crucial was the preferential loss of human

chromosomes from the hybrids, allowing the isolation of clonal hybrid lines with various combinations of human chromosomes against an essentially intact rodent genome. By examining the human gene products of hybrids with various *partial* human genomes the gene products can be associated with specific chromosomes. The mechanisms underlying this chromosome loss are not clearly understood (see Handmaker, 1973, for review), although the loss is apparently divided into two stages with possibly different mechanisms, an early loss which tends to be extensive and which may occur in the first or first few divisions, and a later more gradual loss.

1. Early Chromosome Loss

We will first discuss the early loss seen in primary clonal populations, i.e., uncloned populations derived presumably from a single original hybrid cell in which both intact parental genomes were present. Without regard to possible mechanisms for chromosomal loss, what could we in fact expect from such a hybrid in terms of evolution of its chromosomal constitution? Suppose there are several hundred "vital" genes, the presence of all of which is required for cell survival. We will consider, at least for the sake of discussion, that either a rodent or human gene for a particular function is sufficient. Thus the presence of a complete genome is required, but this could be made up of both rodent and human genes. We will also assume that only one copy of each vital gene is required. Thus monosomy is allowed in our considerations and loss of a gene requires loss of all copies—two for a diploid cell. The presence of a complete genome could be achieved by three alternatives:

1. Retaining an intact rodent genome with permissable loss of any part of or all of the human genome.

2. Retaining an intact human genome with permissable loss of any part of or all of the rodent genome.

3. Retention of a *balanced* complete genome composed of rodent and human chromosomes containing all the vital genes but with permissable loss of any of the other rodent or human chromosomes. Given whatever *mechanism* of chromosome loss the third alternative seems unlikely to occur to us because *no single human chromosome is likely to have the same vital genes as any single rodent chromosome.*

To illustrate, let us consider what would happen if all copies of a particular rodent chromosome containing 10 vital genes are lost. This means that as many as 10 different human chromosomes will be required to be retained. If any of these 10 human chromosomes are subsequently lost the cell will by definition be inviable. If there are 20 vital genes per chromosome the achievement of a balanced heterogenome is even less likely. On the other hand, we can see that if all copies of even one complete chromosome of the human genome are lost, this would probably require the retention of almost all of the rodent genome.

What seems the most likely outcome is that one genome is retained intact (i.e., at least one copy of all vital genes) while the other may be permissibly lost since all the vital genes are in the intact genome (with the important exception of a selected gene which is absent or nonfunctional in the otherwise intact genome: e.g., in HAT the human TK gene will have to be retained in hybrids with a rodent cell lacking TK, but all the other human genes may be lost).

A further consideration is aneuploidy versus euploidy. Many rodent cell lines used to form rodent–human cell hybrids have 1.5–2 times the number of chromosomes found in a normal diploid rodent cell. This means that rodent vital genes are present in a larger number of copies in the hybrids, and it is less likely that, given loss of a certain number of chromosomes, all copies of a particular rodent vital gene will be lost. In fact, it is more likely that all copies of a human vital gene will be lost first, since at least in mapping studies the parental human cells are usually diploid. There is thus a built-in predisposition towards loss of the human genome, and once the direction of loss is established–by loss of all copies of any single human chromosome–the loss of all the rest of the human genome, with the exception of any selected genes, such as TK is allowed. This predisposition cannot account fully for the observed directional loss but may be a contributing factor.

The few observations of loss of rodent and retention of human chromosomes (Jami *et al.*, 1971; Minna and Coon, 1974) may be related to the above considerations (aneuploid human times diploid mouse) or to the lack of *functional* copies of genes "vital" for *in vitro* proliferation in the rodent genome.

It has also been observed that intraspecific hybrids or hybrids between more closely related species (such as rat and mouse) lose chromosomes at a slower rate than seen in rodent–human hybrids (Handmaker, 1973). This could be explained by the achievement of balanced complementing complete genomes, which would be much easier to achieve with intraspecific hybrids or hybrids between closely related species, e.g., the distribution of vital genes would certainly be more similar between rat and mouse than between either rat or mouse and man.

2. Later Chromosome Loss

As explained above, in most rodent-human hybrids human chromosomes are preferentially lost; the loss of any nonselected human chromosome is nonlethal. Once the direction of loss is established in hybrid cells, there are probably two interacting causes of continued loss. One possible cause of later chromosome loss is that cells that have lost human chromosomes have a growth advantage over cells retaining human chromosomes. It has been observed that different human chromosomes are lost from hybrid lines at different rates (Norum and Migeon, 1974). Most explanations for chromosome loss have involved some form of the selective growth advantage argument.

However, it can be shown mathematically (R. Creagan, unpublished results)

that the *irreversibility* of the chromosome loss event (i.e., no progeny of a cell that has lost a particular human chromosome can revert to having the chromosome, so loss is formally analogous to an irreversible mutation) is sufficient to account for the decrease over time (number of generations) in the number of cells retaining human chromosomes in the hybrid population. This can occur even if the cells with and without the human chromosomes have exactly the same generation time (i.e., no selection).

The actual mechanism of late chromosome loss, in either the selective or nonselective model, could be via anaphase lag or nondisjunction. It is important to note that nondisjunction of a human chromosome would result in one daughter cell receiving two copies and the other no copy. By further nondisjunction a cell with two copies could give rise to cells with three or four copies of the specific human chromosome, and so on. This mechanism could readily explain the often observed multiple copies of human chromosomes in hybrid cells (Green *et al.*, 1971; Marcus *et al.*, 1976).

The above mechanisms of selective loss and nonselective irreversible loss are quite adequate to explain the gradual, progressive later loss of human chromosomes from rodent-human hybrids. Although the mechanisms involved in the early loss-mediated establishment of directionality are not yet understood, it is clear that once the directionality is established, at least later chromosome loss can be explained adequately by mechanisms which are known to occur. It should be pointed out, however, that there have as yet been no rigorous demonstrations that loss does in fact occur by the mechanisms proposed above, and similarly, more speculative mechanisms have not been ruled out.

F. Selective Systems

Since genetic events often occur at low frequencies, selective systems are key elements in genetic analysis. The uses of selective systems in human gene mapping include mutant selection (Siminovitch, 1976; DeMars, 1974; Thompson and Baker, 1973), hybrid cell selection, selection of specific chromosomes or chromosome regions, and gene and chromosome transfer experiments.

There are several types of selection which we can discuss. Selection may simply imply the physical enrichment for, or isolation of, cells with a certain property. Visual selection using morphological or immunological criteria, sib-selection methods (Marin, 1969; Rosenstraus and Chasin, 1975), and use of fluorescence-activated cell sorters (Hulett *et al.*, 1973; Kreth and Herzenberg 1974) would fall into this category.

Selection may also mean that the culture medium is constructed in such a way that cells with a certain property have a different potential for growth or survival. Selection of this type can be positive (cells with a certain property survive or grow more rapidly) or negative (cells with a particular property are

killed or grow more slowly). Selection can be relative—different rates of proliferation for cells with particular phenotypes, or absolute—cells with or without a certain phenotype are killed. The phenotypes selected can be classified as dominant (presence of a phenotype is selected for or against) or recessive (absence of a phenotype is selected for or against). It should be noted that depending upon the selective conditions, the same phenotype can be selected for or against.

There are two main functions of selective systems in human gene mapping. The most basic is the selection of hybrids against a background of parental cells. A second important function is to select for specific gene products and thus select for the presence of particular chromosomes or chromosome fragments containing the specific selected gene.

A system which has received wide use is the hypoxanthine-aminopterin-thymidine (HAT) system (Hakala, 1957; Hakala and Taylor, 1959; Szybalski *et al.*, 1962; Littlefield, 1964). Purine analogues such as 8-azaguanine and 6-thioguanine require conversion to nucleotides by the enzyme hypoxanthine phosphoribosyltransferase (HPRT) to exert their toxic effects. Cell lines were derived which were resistant to 8-azaguanine and 6-thioguanine; these lines did not express HPRT. This lack of HPRT and consequent resistance to 8-azaguanine occurs naturally in cells of individuals with the Lesch-Nyhan syndrome. Similarly, the thymidine analogue bromodeoxyuridine (BUdR) can be used to select for cells lacking thymidine kinase (TK). Cells lacking HPRT or TK can survive since the normal *de novo* pathways for purine and thymidine synthesis are intact.

Methotrexate, a drug used in cancer chemotherapy, and the closely related aminopterin (which is too toxic for clinical use), act as potent inhibitors of dihydrofolate reductase, an enzyme crucial to both *de novo* purine and thymidylate synthesis. Cells treated with aminopterin can survive if hypoxanthine and thymidine are added to the medium*, but only if they retain the respective "salvage" enzymes HPRT and TK. Cells blocked with aminopterin are thus conditionally auxotrophic for thymidine and an exogenous purine source.

Littlefield (1964) first utilized the HAT system for hybrid cell selection. He co-cultivated two cell lines, one lacking HPRT but retaining TK, and the other lacking TK and retaining HPRT, in medium containing hypoxanthine, aminopterin, and thymidine. Since both HPRT and TK are required for survival in HAT, both the $TK^-/HPRT^+$ and the $TK^+/HPRT^-$ parental strains were killed. Hybrid cells which retained the TK from one parent and the HPRT from the other parent survived. This prototype biochemical selection system has proved extremely useful for hybrid selection.

*Note: Glycine is also required since aminopterin also blocks glycine synthesis, and this selective system is sometimes referred to as the THAG system. Glycine, however, is normally a component of the culture medium, and the system is most commonly referred to as the HAT system.

A problem with the HAT system is that the parental cells must be either $HPRT^-$ or TK^-, and this usually requires a prior selective process. For human gene mapping studies it was felt that the human cell should be as "normal" as possible and should therefore have a normal or well-characterized karyotype. Prior selection may result in an aneuploid cell line, however. Several solutions to this problem have been found. The first and simplest solution is to use primary lymphocytes as the human parent. These cells do not proliferate indefinitely and also do not attach to the substrate and thus can be removed easily. Alternatively, slowly growing, semisenescent fibroblasts can be used, but the problems of karyotypic abnormalities in senescent cells have to be considered. Human cells naturally lacking HPRT, derived from Lesch-Nyhan patients, can also be used without prior selection.

A recent solution has come from the naturally occurring difference in sensitivity of rodent and human cells to the cardiac glycoside ouabain (Baker *et al.*, 1974). Human cells are much more sensitive to ouabain than rodent cells, but the hybrids are apparently as resistant as the parental rodent cells (i.e., resistance appears to be dominant). Hybrids can thus be readily obtained from a cross of TK^- or $HPRT^-$ rodent cells with human cells by selecting in HAT–ouabain medium. Rodent cells also have a greatly increased resistance to diphtheria toxin as compared to human cells (Creagan *et al.*, 1975). In this system, however, sensitivity is dominant in the hybrids. The sensitivity gene has been localized to human chromosome 5 (Creagan *et al.*, 1975). A particularly useful aspect of this system is that the preferential loss of human chromosomes is assured (see Section III,E).

A second main use of selective systems is in gene mapping. If a human phenotype can be selected for or against in hybrid cells, one can hopefully establish a correlation between the respective presence or absence of the selected phenotype and the presence or absence of a specific chromosome. For example, the HAT selective system was used to establish the assignment of the gene for TK to human chromosome 17 (Miller *et al.*, 1971; Boone *et al.*, 1972). TK^- mouse cells (selected with BUdR) were fused with human cells in HAT medium, and the hybrids were examined for the presence of a common human chromosome, which would presumably carry the TK gene The hybrid lines were then back-selected with BUdR to show the loss of the proposed chromosome concurrent with the loss of TK. The possibility of selecting negatively (using BUdR) and positively (using HAT) for the same phenotype is obviously very useful in establishing a phenotype–chromosome association, but either positive or negative selection alone can also be useful. For example, selection against the diphtheria toxin sensitivity gene allowed the assignment of this gene to chromosome 5, based on the unique loss of chromosome 5 in the cells surviving toxin selection (Creagan *et al.*, 1975).

The selective systems for HPRT and TK exemplify a number of recently

devised drug-resistance/conditional auxotrophy systems (Chan *et al.*, 1975; Chan and Creagan, 1976; Dechamps *et al.*, 1974; Medrano and Green, 1974; Kusano *et al.*, 1971; Tischfield and Ruddle, 1974). Most of the drugs have been developed as possible cancer chemotherapeutic agents and are toxic to cells in culture after conversion by an enzyme not ordinarily necessary for cell survival *in vitro*. Thus cells can be selected which lack the specific conversion enzyme, e.g., 2-fluoroadenine can be used to select for cell lines lacking adenine phosphoribosyltransferase (APRT), which converts the nonlethal 2-fluoroadenine to the toxic nucleotide form (Bennett *et al.*, 1966; Kusano *et al.*, 1971; Tischfield and Ruddle, 1974). By the use of hadacidin or alanosine which block the conversion of IMP to AMP (Fig. 2), cells are made conditionally auxotrophic for an exogenous AMP source. Thus by first selecting APRT$^-$ rodent cells with 2-fluoroadenine, and then selecting for rodent–human hybrid cells with alanosine-adenine (Kusano *et al.*, 1971; Tischfield and Ruddle, 1974), or

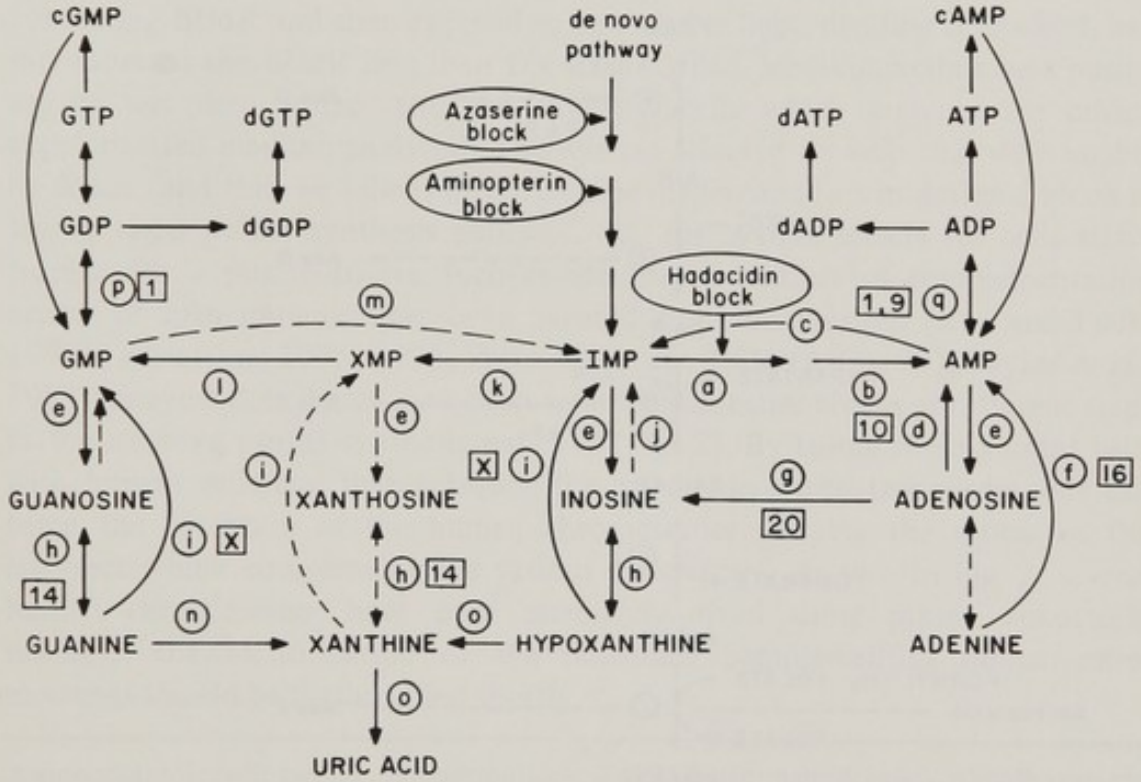

Fig. 1. Purine interconversions: (a) adenylosuccinate synthetase (EC 6.3.4.4); (b) adenylosuccinate lyase (EC 4.3.2.2); (c) AMP deaminase (EC 3.5.4.6); (d) adenosine kinase (EC 2.7.1.20); (e) 5′-nucleotidase (EC 3.1.3.5); (f) adenine phosphoribosyltransferase (EC 2.4.2.7); (g) adenosine deaminase (EC 3.5.4.2); (h) purine nucleoside phosphorylase (EC 2.4.2.1); (i) hypoxanthine-phosphoribosyltransferase (EC 2.4.2.8); (j) inosine kinase (postulated enzyme); (k) IMP dehydrogenase (EC 1.2.1.14); (l) GMP synthetase (EC 6.3.5.2); (m) GMP reductase (EC 1.6.6.8); (n) guanine deaminase (EC 3.5.4.3); (o) xanthine oxidase (EC 1.3.2.3); (p) guanylate kinase (EC 2.7.4.8); (q) adenylate kinase (EC 2.7.4.3). ◄——►, known mammalian pathways; ◄– – –►, questionable or absent in mammalian cells; ○, enzyme code letters (listed above); □, human chromosome assignments.

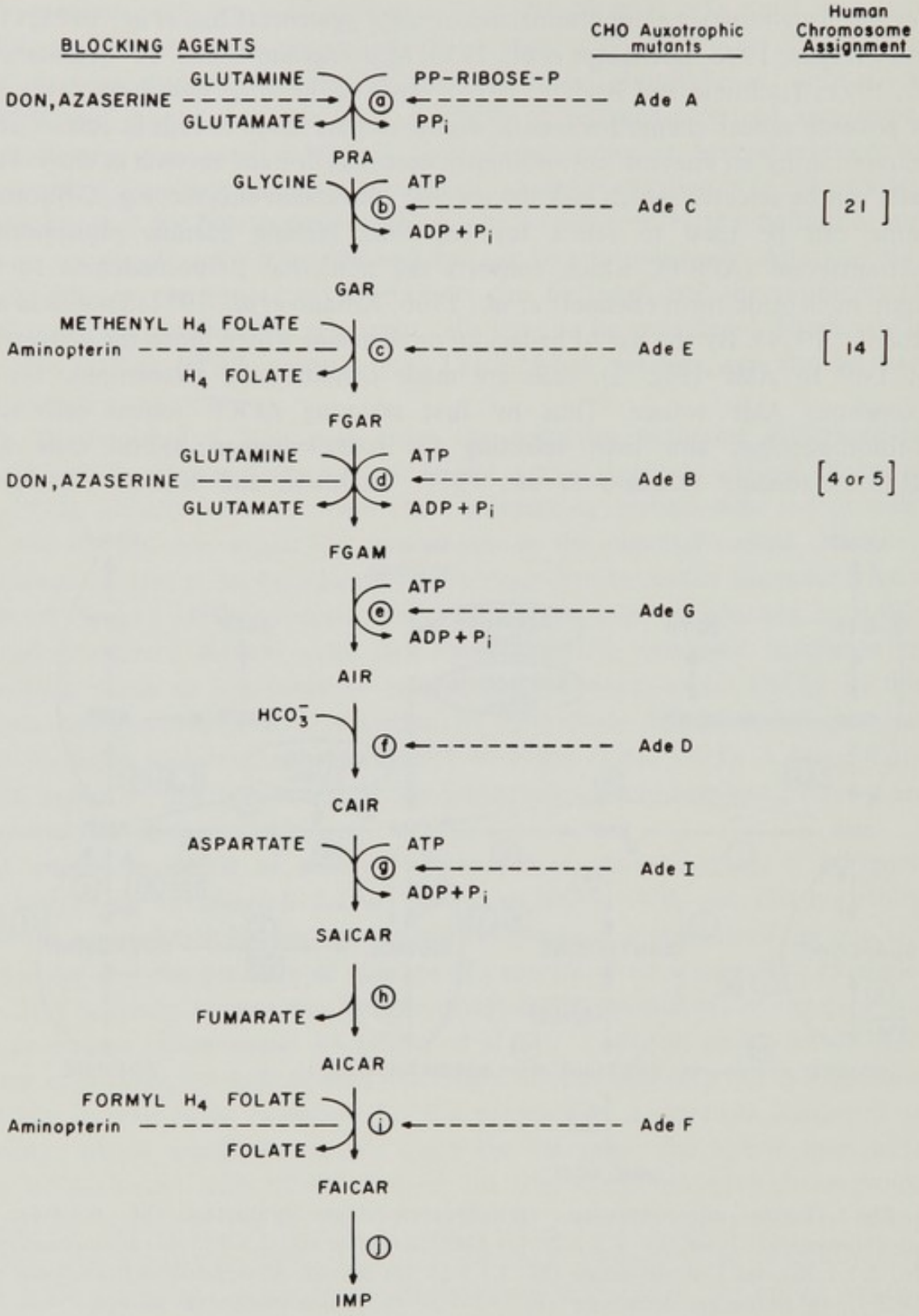

Fig. 2. Auxotrophic mutants in the *de novo* purine synthesis pathway. Abbreviations for intermediates in order of synthesis: PRA (phosphoribosylamine); GAR (glycineamide ribonucleotide); FGAR (formylglycineamide ribonucleotide); FGAM (formylglycineamidine ribonucleotide); AIR (aminoimidazole ribonucleotide); CAIR (aminoimidazolecarboxylate ribonucleotide); SAICAR (aminoimidazole-*N*-succinocarboxamide ribonucleotide); AICAR

azaserine-adenine (Kahan *et al.*, 1974) the human chromosome 16 which carries the APRT gene can be selectively fixed in the hybrid cells (Fig 1).

An even broader method is the selection of conditionally lethal recessive mutants by use of a system similar to the penicillin selection system used to select mutants in bacteria. Broadly, the system involves adjusting the culture conditions so that the mutant cells are not dividing, and introducing a cytotoxic agent that kills dividing cells. Such systems have used BUdR/light, ara-C, or tritiated thymidine as the lethal agent (Puck and Kao, 1967; Thompson and Baker, 1973). The cytotoxic agent is removed after an appropriate period, and conditions adjusted so that the mutant cells can proliferate. This procedure may have to be repeated one or more times to enrich the population adequately in mutant cells.

An excellent example of this system is the isolation of nutritional auxotrophs by T. T. Puck, F.-T. Kao and their colleagues. After first removing all exogenous purine sources from the culture media, cells were cycled through a medium containing BUdR and then exposed to near-visible light; dividing cells which had incorporated the BUdR into their DNA were killed. Medium containing a purine source was then added, and the surviving cells which grew in the purine supplemented medium analyzed. This system selected for cells that were unable to divide (and thus be killed) in purine-free media, due to a mutational block in the *de novo* purine synthesis pathway, i.e., the system selects for cells auxotrophic for a purine source such as adenine. A number of complementation classes of such mutants have been isolated and characterized (Kao and Puck, 1972a; Patterson, 1975, 1976; Patterson *et al.*, 1974 (see p. 97); Taylor *et al.*, 1971); enzyme deficiences have been found which cause blocks at different steps in the *de novo* purine synthesis pathway (Fig 2). By fusing these mutant cells with human cells and then selecting the hybrids in purine-free media, one can force the retention of the human chromosomes carrying the genes for the enzymes which complement the various deficiencies. As seen in Fig 2, several human chromosomes have been selectively fixed using purine auxotroph mutants; the identification of the remaining complementing human chromosomes should be forthcoming shortly.

(aminoimidazolecarboxamide ribonucleotide); FAICAR (formamidoimidazolecarboxamide ribonucleotide); IMP (inosine monophosphate). Enzymes as indicated above by circled letters: (a) PP-ribose-P amidotransferase, EC 2.4.2.14; (b) GAR synthetase, EC 6.3.1.3; (c) GAR formyltransferase, EC 2.1.2.2.; (d) FGAM synthetase, EC 6.3.5.3; (c) AIR synthetase, EC 6.3.3.1; (f) AIR carboxylase, EC 4.1.1.21; (g) SAICAR synthetase, EC 6.3.2.6; (h) AMPSL (adenylosuccinate lyase), EC 4.3.2.2; (i) AICAR formyltransferase, EC 2.1.2.3; (j) IMPCH (inosinate cyclohydrolase), EC 3.5.4.10.

NOTE: Blocking agents refers to agents that block the *de novo* pathway at the indicated steps. *CHO auxotrophs* refers to auxotrophic mutants isolated in the CHO cell line (Kao and Puck, 1974, 1975). *Human chromosome assignment* indicates the human chromosome which carries the enzyme complementing the auxotrophy.

TABLE II
Selective Systems Specific for Particular Human Chromosomes

Human chromosome	Phenotype	Selective agents or conditions	
		Positive	Negative
1[a]	Uridine-cytidine kinase	Adenosine-uridine[b]	^{3}H-uridine[b] or 5-fluorouridine[b]
3[c]	Phenotype complementing heat-sensitive mutant	Nonpermissive temperature[c]	Nonpermissive temperature + cytotoxic agents[c]
4 or 5[d]	Formylglycineamidine ribonucleotide synthetase	Purine-free media[d]	Purine-free media + cytotoxic agents[d]
5[e]	Diphtheria toxin sensitivity	FACS*	Diphtheria toxin[e]
6[f]	HLA cell surface antigen	FACS*	Antisera + complement[f]
7[g]	Human cell surface antigen	FACS*	Antisera + complement[g]
8[h]	Enzyme complementing glycine auxotroph[s]	Glycine-free media[s]	Glycine-free media + cytotoxic agents[s]
10[i]	Adenosine kinase	Alanosine + adenosine + uridine[j]	6-Methylthiopurine ribonucleotide (MSIR)[k,l] or tubercidin[k,l] or 2-fluoradenosine (FAR)[k,l] or adenosine[m]
10[n]	Glutamate γ-semialdehyde synthetase	Proline-free media	Proline-free media + cytotoxic agents[n]
11[o,p,q,r]	Human cell surface antigen	FACS*	Antisera + complement[o,p,q,r]
12[t]	Serine hydroxymethylase	Glycine-free media[s,t]	Glycine-free media + cytotoxic agents[s,rt]
14[h]	Glycineamide ribonucleotide formyl transferase	Purine-free media[u]	Purine-free media + cytotoxic agents[u]
15[v,w,x,y]	β_2-Microglobulin	FACS*	Antisera + complement[v,w,x,y]
16[z,aa]	Adenine phosphoribosyltransferase	Alanosine-adenine[z,bb] or azaserine-adenine[aa]	2,6-Diaminopurine[z,aa,bb] or 2-fluoroadenine[z,aa,bb]

17[cc]	Galactokinase	Media containing galactose[cc]	2-Deoxygalactose[dd]
17[ee,ff]	Thymidine kinase	Hypoxanthine-aminopterin-thymidine (HAT)[gg,hh]	Bromodeoxyuridine (BUdR)[gg,hh]
19[ii]	Polio virus receptor	FACS*	Polio virus[ii]
21[kk]	Glycineamide ribonucleotide synthetase	Purine-free media[u]	Purine-free media + cytotoxic agents[v]
X[o]	Hypoxanthine phosphoribosyltransferase	Hypoxanthine-aminopterin-thymidine (HAT)[gg,hh]	8-Azaguanine[gg,hh] or 6-thioguanine[gg,hh]
X[ij]	Human cell surface antigen	FACS*	Antisera + complement[ij]

*It should be possible to select positively for these cell-surface phenotypes through the use of the fluorescence-activated cell sorter (FACS) (Hulett *et al.*, 1973; Kreth and Herzenberg, 1974).

[a] L. Medrano, personal communication.
[b] Medrano and Green, 1974.
[c] Ming *et al.*, 1976.
[d] Kao and Puck, 1972a.
[e] Creagan *et al.*, 1975.
[f] Van Someren *et al.*, 1974.
[g] Aden and Knowles, 1976.
[h] F.-T. Kao, personal communication.
[i] Klobutcher *et al.*, 1976.
[j] Chan and Creagan, 1976.
[k] Bennett *et al.*, 1966.
[l] Chan *et al.*, 1973.
[m] McBurney and Whitmore, 1975.
[n] Jones, 1975.
[o] Nabholz *et al.*, 1969.
[p] Puck *et al.*, 1971.
[q] Buck and Bodmer, 1975.
[r] Jones *et al.*, 1975.
[s] Kao *et al.*, 1969.
[t] Jones *et al.*, 1972.
[u] Patterson, 1975.
[v] Goodfellow *et al.*, 1975.
[w] Faber *et al.*, 1976.
[x] Solomon *et al.*, 1976.
[y] Smith *et al.*, 1975.
[z] Tischfield and Ruddle, 1974.
[aa] Kahan *et al.*, 1974.
[bb] Kusano *et al.*, 1971.
[cc] Elsevier *et al.*, 1974.
[dd] Thirion *et al.*, 1976.
[ee] Miller *et al.*, 1971.
[ff] Boone *et al.*, 1972.
[gg] Szybalski *et al.*, 1962.
[hh] Littlefield, 1964.
[ii] D. A. Miller *et al.*, 1974.
[jj] Buck *et al.*, 1976.
[kk] Jones *et al.*, 1976.

Similar systems can be derived which select for human chromosomes carrying genes complementing other nutritional auxotrophs (Kao and Puck, 1972b, 1974, 1975; Naylor *et al.*, 1976; Siminovitch, 1976; Thompson and Baker, 1973), mutants in carbohydrate utilization (Burns *et al.*, 1976), and respiratory mutants (Ditta *et al.*, 1976). Temperature-sensitive mutants can be selected by procedures similar to those outlined for nutritional auxotroph mutants (Patterson *et al.*, 1976; Thompson *et al.*, 1971, 1975). The actual basis of some of these temperature-sensitive mutants is known (Fenwick and Caskey, 1975; Thompson *et al.*, 1975) but temperature-sensitive mutants of unknown basis can be useful for selection of the human chromosome carrying the gene for the function which complements the temperature-sensitive mutation (Ming *et al.*, 1976).

As mentioned above, the ability to select for the loss of a specific human chromosome from a hybrid may be useful for mapping. Human chromosomes carrying genes for phenotypes which render the hybrid cells sensitive to specific cytotoxic agents can be selected against. Thus the gene on human chromosome 5 for diphtheria toxin sensitivity (Creagan *et al.*, 1975) and the gene on chromosome 19 for a poliovirus receptor allow the negative selection of these two chromosomes in human-rodent hybrids, since the rodent parental cells are naturally resistant to both these agents. Antisera to specific human cell-surface antigens can be similarly used as chromosome-specific cytotoxic agents (Table II). Since the basis for sensitivity for this last class of selectable phenotypes resides on the cell surface, it is hoped that these phenotypes will be susceptible to positive selection by use of a fluorescence-activated cell sorter (Hulett *et al.*, 1973; Kreth and Herzenberg 1974). In addition to naturally occurring differences, rodent cells resistant to the actions of toxins and lectins can be selected (Gottlieb *et al.*, 1974; Stanley *et al.*, 1975; Stanley and Siminovitch, 1976). Human chromosomes which carry genes for products restoring sensitivity to the toxins to human-rodent hybrid cells should be susceptible to negative selection.

A further approach with potential for human gene mapping, involving prior selection of human cells, is the selection of human chromosomes carrying genes for dominant resistance phenotypes. For example, human cells selected for resistance to α-amanitin have an altered RNA polymerase II which is resistant to inhibition by α-amanitin (Chan *et al.*, 1972; Buchwald and Ingles, 1976). Since this resistance is dominant, it should be possible to select for the retention of the human chromosome carrying the RNA polymerase II gene in hybrids between resistant human cells and sensitive cells. Human cells resistant to methotrexate due to an altered dihydrofolate reductase could be used in a similar manner (Flintoff *et al.*, 1976).

Although selective systems have been important to somatic cell genetic systems to date, the selective fixation and elimination of specific genes and chromosomes are even more vital to the successful use of the newer methods of gene mapping such as the method of Goss and Harris (1975), and gene and

chromosome transfer. The current rapid expansion in selection methods should prove extremely useful in somatic cell genetics, much as similar selective systems have been a basic tool in studying and using prokaryotic genetic systems.

G. Mapping Strategies

As the historical account above indicates, the qualities of rodent–human hybrids that make them useful for human gene mapping are

1. Preferential or unilateral loss of human chromosomes (in most cases).
2. Easy detection of human phenotypes and differentiation from their rodent counterparts.
3. Reliable identification of human chromosomes in interspecific hybrids.

Using these properties, several approaches can be followed to determine chromosome assignments with somatic cell hybrids. These include synteny testing, assignment by selection, and assignment by association of nonselectable genes. A refinement of assignment by association is the clone panel system (Creagan and Ruddle, 1975). These same strategies can be used to assign genes to regions of individual chromosomes. Goss and Harris (1975) developed an extension of the regional assignment method which may provide a powerful tool for assignment of genes to small chromosomal regions. Gene and chromosome transfer should also provide a potential means of high resolution mapping.

1. Synteny Testing

Synteny testing is based on the premise that genes which are located on the same chromosome will be retained and lost concordantly from hybrid cells. Thus, even before reliable chromosome identification methods were available, a number of gene pairs or groups were determined to be syntenic by observing the pattern of retention and loss in a large number of independently derived hybrid cell lines. If one member of a synteny group is assigned to a chromosome, the other members can then be presumptively assigned to the same chromosome. As reliable chromosome identification methods are now available, synteny testing should always be supported by chromosomal analysis, to rule out the possibility of preferential concordant retention or loss of a pair of chromosomes which could give rise to a false indication of synteny as well as to rule out the existence of extensive chromosome rearrangements which would obscure a true syntenic relationship.

2. Assignment by Selection

The first assignments of genes to chromosomes using rodent–human hybrids relied on the ability to select for the genes in question. Thus, the assignment of the TK gene to chromosome 17 was facilitated by the isolation of reduced hybrids which contained only chromosome 17 in common (Boone *et al.*, 1972;

Migeon and Miller, 1968; Miller *et al.*, 1971; Weiss and Green, 1967). The retention of this chromosome under conditions of selection for TK was evidence for assignment. By back-selecting these cells with BUdR, which selects against the TK gene, it could be demonstrated that loss of chromosome 17 correlated with loss of the TK gene.

The assignment of a gene which is syntenic with a selectable gene can also be facilitated since retention or loss of the nonselectable syntenic gene can be shown to correlate with selection for or against the selectable gene. Thus, the assignment of the gene for galactokinase was supported by the demonstration that the galactokinase gene was retained in cells selected for TK and lost in cells selected for the loss of TK (Elsevier *et al.*, 1974).

Negative selection alone can also be useful. Creagan *et al.* (1975) supported their assignment of a gene for diphtheria toxin sensitivity by culturing hybrid lines showing high sensitivity to diphtheria toxin in media containing the toxin. While the sensitive lines had shown a high level of chromosome 5 prior to toxin treatment, the surviving cells uniformly had lost chromosome 5. The levels of other human chromosomes in sensitive lines and these lines after toxin treatment were relatively unchanged. The assignment of the gene for hexosaminidase B (Hex B) (Gilbert *et al.*, 1975) was also strengthened by the demonstration that cell lines sensitive to toxin had Hex B activity, but that these lines after toxin treatment had lost Hex B. Again, levels of other human enzymes were not affected by the toxin treatment.

3. Assignment by Association

The assignment of nonselectable genes relies on observation of retention or loss of the expression of the phenotype coded by the gene(s) concordant with the respective retention or loss of a specific chromosome or chromosomes. Assignment of a gene by this method begins with the observation of an association of expression of a phenotype with presence of a specific chromosome. One or more positive hybrid lines are then subcloned and the phenotype-chromosome association examined in the subclones. Ideally it can be shown that lines retaining only a single human chromosome express the phenotype in question, thus providing unambiguous assignment of a structural gene to that chromosome. The reliability of this method is limited only by the reliability of the phenotype assays and chromosome identification methods used. Discrepant clones should be carefully examined for chromosome rearrangements. The percentage of hybrid cells which contain a specific chromosome will obviously affect detection of an associated phenotype, especially if the phenotype assay is performed on extracts of cell populations. An effort should therefore be made to use cell lines in which all chromosomes present are in a high percentage of cells. Based on experience with this approach, a reliable system utilizing a

relatively small number of cell lines has been developed, which we have termed the *clone panel* system (Creagan and Ruddle, 1975).

4. Clone Panel

As discussed above, the assignment of a human gene to a particular chromosome by association requires that the gene be present and expressed in the hybrid lines examined in such a way that the pattern of expression may be correlated uniquely with the presence of a specific human chromosome. The minimum number of ideal hybrid lines necessary to meet these requirements is surprisingly small. If we consider a subset of 8 human chromosomes, three hybrid lines with the chromosomal patterns as shown in Fig. 3 would suffice. For example, if a phenotype were expressed in all three lines it could be assigned to chromosome 1 (Fig. 3, column 1); if expressed in lines A and C, but not B (shaded column), assignment could be made to chromosome 3, and so on. The number of unique patterns possible in a panel of n lines is in fact identical to the total number of binary numbers with a maximum of n digits. Thus for 3 clones the number of patterns, as seen in Fig. 3, is 2^3 or 8 and for n clones is 2^n patterns. The number of different human chromosomes is 24 (22 autosomes and the X and Y). One can provide 24 patterns with a minimum number of 5 clones ($2^5 = 32$ patterns). It is thus theoretically possible to construct a panel of five lines which will have unique patterns for all 24 different human chromosomes, and therefore a unique pattern of phenotypic expression for genes located on each chromosome. While it may be unlikely that we will be able to derive lines easily with the *ideal* chromosome distribution required for the use of a minimum number of lines, we can certainly approach this minimum number. We have termed systems using small numbers of carefully selected and characterized lines *clone panels* to emphasize the clonal origin of the lines used (Creagan and Ruddle, 1975).

Having determined that a small number of hybrid lines can at least theoretically be used for gene mapping, we can consider the practical problems and limitations involved in deriving and using such a system. Our goal is to obtain a

		HUMAN CHROMOSOMES							
		1	2	3	4	5	6	7	8
HYBRID CLONES	A	+	+	+	+	−	−	−	−
	B	+	+	−	−	+	+	−	−
	C	+	−	+	−	+	−	+	−

Fig. 3. A three clone panel capable of discriminating eight chromosomes. A phenotype found in clones A and C, but not B (shaded column) could be assigned to chromosome 3.

small number of well-characterized lines which can be expanded to give a large population of cells retaining the desirable characteristics of the original lines. Since the characteristics of the hybrid lines will be determined largely by those of the nonhuman parental line, the choice of the nonhuman parental line is critical.

The following criteria are among those that should be considered in selecting parental and hybrid lines:

1. Rapid growth rates and ease of culture.
2. Ease of identification of human chromosomes and discrimination from chromosomes of the nonhuman parent.
3. Expression and detection of human phenotypic markers.
4. Ease of derivation of hybrid lines.
5. Stability of human chromosome complements.
6. Possibility of relatively unlimited expansion.
7. "Specific activity" of phenotypes.

Most hybrids used for human gene mapping have had as the nonhuman parent heteroploid, permanent cell lines of either mouse or Chinese hamster origin (Sell and Krooth, 1972). Most such rodent–human combinations readily satisfy criteria 1 through 6 above. We shall discuss more fully the selection of hybrid lines with high "specific activity" for human phenotypes.

5. "Specific Activity" of Human Phenotypes and DNA

Detection of human phenotypes, particularly detection of human enzymes, or direct detection of human genes using Cot analysis (Deisseroth *et al.*, 1976), depends in large part on the "specific activity" of the human genes or phenotypes in the hybrid cells or cell populations. While semi-purification of enzymes or target DNA would certainly enhance the sensitivity of an assay, this could prove difficult or laborious in practice. It is therefore desirable to obtain the highest possible ratio of enzyme protein to total protein, or target DNA to total DNA. These ratios are affected by the number of copies of the gene per cell, the percentage of total cells in which the desired gene is to be found, and the total amount of protein or DNA per cell; the latter totals will be largely determined by the size of the nonhuman parental genomes.

The quantitative expression of a number of phenotypes such as some enzymes, structural proteins, or cell surface markers reflects a linear relationship to the number of genes for the particular phenotype present in the cell (see Section II,D). In man, for example, cell strains trisomic for chromosome 16 have approximately 1.5 times as much adenine phosphoribosyltransferase (APRT) per cell as normal diploid cell strains (which are disomic for 16), while other enzymes tested, and presumably total cell protein, were approximately the same in both cell types (Marimo and Gianelli, 1975). We would expect a higher

specific activity in trisomy 16 cells for phenotypes coded by genes on 16 and also for chromosome 16-specific DNA.

The specific activity of human DNA will depend on the ratio of the human target DNA to the total DNA. In cells in culture the aneuploidy of the permanent cell lines used as nonhuman parents may give rise to cell lines with 1.0 (pseudodiploid) to 2.0 (pseudotetraploid) or more genome equivalents, where we define one genome equivalent as the amount of DNA in a normal diploid cell. In terms of specific activity, we would prefer to use as nonhuman parents, cell lines that were closer to pseudodiploid than pseudotetraploid.

In the isolation of hybrid cell lines we find lines with one, two, or three times the modal number of chromosomes of the nonhuman parental stem line; we call these, respectively, 1S, 2S, and 3S hybrids. Thus, a 2S hybrid from a nonhuman parental line with 1.5 genome equivalents per cell would have 3 genome equivalents of nonhuman DNA per cell. While it is a possibility that there exist differences in "stability" or loss rate of human chromosomes from hybrids of different S number (Attardi and Attardi, 1972) it is obvious that, other factors aside, our highest specific activity of human DNA would be obtained in 1S hybrids.

Cell lines with a high percentage of cells with at least one copy of a particular chromosome would also be desirable. This goal can be approached by the use of nonhuman parental lines in which human genes can be selected. Use of selection ensures that the percentage of cells with at least one copy of a chromosome approaches 100%, and also ensures that populations expanded under selective conditions will retain this high percentage. Also the isolation of cell lines with an average of more than one copy of specified genes or chromosomes per cell would depend largely on selection systems. Hybrids with high "specific activities" for particular chromosomes could be used alone for detection of phenotypes where the sensitivity of the assay systems was low, or alternatively could potentially reduce the number of cells needed for any one assay. Of particular interest for clone panels is the possibility that high "specific activity" lines could be mixed to give an ideal clone panel. If we set as our minimum acceptable level an average of 0.5 copies per cell of a particular chromosome against a 1S nonhuman background, we would require a 2S hybrid to possess an average of 1.0 copies of the particular chromosome per cell, which effectively rules out the use of 2S hybrids under nonselective conditions (under our arbitrary standard). However, if we could isolate cell lines having an average of 3 copies of a chromosome per cell against a 1S background, we could in fact *mix* extracts of up to six of these lines and still have the same specific activity for human DNA and protein phenotypes, as we would have had in the single 2S line with 1.0 copies per cell. It is clear that availability of such high "specific activity" lines could greatly facilitate the construction of an ideal clone panel.

6. Regional Localization

While the assignment of a gene to a specific chromosome is the usual first step in gene mapping, a number of methods have been developed to subsequently localize the assigned genes to specific regions of chromosomes and to determine gene order. *In situ* hybridization, which is discussed extensively in Chapter 3, is certainly the most direct way to establish correspondence of a gene with a chromosome region, but the potential of this method for determining high-resolution localization is still not clear.

Conventional family linkage studies can determine relative gene order by three-point mapping and can also give a measure of intergene distance in terms of meiotic recombination fractions. In addition, linkage to inherited chromosome markers can also localize genes along particular chromosomes (Robson *et al.*, 1969; Magenis *et al.*, 1970). Deletions or additions of genetic material can also be useful for gene localization, as in the localization of the gene for acid phosphatase to a deleted segment of human chromosome 2 (2q21-2qter) (Ferguson-Smith *et al.*, 1973). (See Section II,D.)

Somatic cell hybridization can establish subchromosomal localization through correlation of phenotypic expression with the presence of particular chromosome regions. One way to approach this is to utilize human parental cells carrying chromosome translocations or deletions. For instance, as mentioned above, hybrids made with human cells carrying a translocation of the long arm of the X chromosome to chromosome 14 allowed Ricciuti and Ruddle (1973a,b) to localize the genes for G6PD, HPRT, and PGK to the long arm of the X chromosome. A number of investigators using various additional translocations involving the X chromosome have confirmed this localization and determined the order of the genes along the X chromosome (cf. Bergsma, 1974, 1975a, 1976). A growing collection of human cell strains carrying various translocations has been established and made available to investigators at the Institute of Medical Research in Camden, New Jersey (Greene, 1975). The most efficiently used translocations are those which contain a selectable gene such as HPRT (translocations with the long arm of the X chromosome) or TK (translocations with the long arm of chromosome 17). As the number of selective systems increases, an increasing number of translocation-carrying human cell strains will become useful.

Another approach is to utilize induced or spontaneous translocations or deletions arising in the hybrid cells (Boone *et al.*, 1972; McDougall *et al.*, 1973; Burgerhout *et al.*, 1973). Boone *et al.* (1972) took advantage of a spontaneous translocation of the long arm of chromosome 17 to a mouse chromosome to localize the gene for thymidine kinase to 17q. Using deletions induced by irradiating hybrid cells, Burgerhout *et al.* (1973) determined the order of a number of genes on chromosome 1. Adenovirus 12 has been used to induce

deletions in a specific region of the long arm of chromosome 17, thus providing evidence for the localization of the genes for thymidine kinase (McDougall *et al.*, 1973) and galactokinase (Elsevier *et al.*, 1974) to the 17q21-22 region.

The method of quantitative mapping in hybrids developed by Goss and Harris (1975) and discussed further in Section V,A, will hopefully prove highly useful for regional localization. In addition, the new methods of gene and chromosome transfer, and Cot analysis of hybrid cells, also discussed in Section V, have great potential for high resolution mapping.

IV. THE HUMAN GENE MAP

The list of human genes assigned to chromosomes as given in Table III is up to date as of the Baltimore Conference (Bergsma, 1976), which was held in October, 1975. References for these assignments are too numerous to be included here but may be found in the Baltimore Conference (Bergsma, 1976) and in the proceedings of two earlier conferences on human gene mapping, the New Haven Conference (Bergsma, 1974) and the Rotterdam Conference (Bergsma, 1975a). Two recent reviews of human gene mapping are those of McKusick and Chase (1973), which emphasize the results from family studies, and Ruddle and Creagan (1975) which emphasize results from somatic cell hybrids.

No gene symbols are given in Table III, except for the blood group symbols, because symbols for other genes are in a state of flux. Refer to the Baltimore Conference (Bergsma, 1976) for current recommendations.

V. NEW MAPPING METHODS

A. Quantitative Mapping Using Irradiated Cells

An extension and refinement of the methods discussed above may permit the use of somatic cell hybrids to determine a high resolution gene map with both gene order and quantitative intergene distance being readily determined. The method developed by Goss and Harris (1975; Goss, 1976) uses human normal diploid parental cells which have been subjected to carefully quantitated doses of γ-irradiation. The irradiated cells are immediately fused with rodent parental cells and the cells plated out in selective medium. If the rodent cells are $HPRT^-$, and the cells are selected in HAT medium, the human HPRT gene will be selected for. Other X-linked human markers will be retained at a frequency which is a function of both the distance from the HPRT gene and the dose of irradiation. While this method has been used to date only for determining the order of X-linked markers, the results have been consistent with the order determined by using translocations.

TABLE III
Human Gene Assignment

Locus	Status[a]	McKusick number[b]	EC number
Chromosome 1			
Adenovirus 12: Chromosome 1 modification site	P	10293	–
Adenylate kinase-2	C	10302	2.7.4.3
Amylase-1 (salivary)	C	10470	3.2.1.1
Amylase-2 (pancreatic)	C	10465	3.2.1.1
Duffy blood group (F)	C	11070	–
Elliptocytosis-1 (*Rh* linked)	C	13050	–
Enolase (phosphopyruvate hydratase)	C	17245	4.2.1.11
α-L-Fucosidase	P	23000	3.2.1.51
Fumarate hydratase-1 (fumarase)	C	13685	4.2.1.2
Guanylate kinase-1	C	13927	2.7.4.8
Guanylate kinase-2	P	13928	2.7.4.8
Peptidase C	C	17000	3.4.11.-
6-Phosphogluconate dehydrogenase	C	17220	1.1.1.44
Phosphoglucomutase-1	C	17190	2.7.5.1
Rhesus blood group (*Rh*)	C	11170	–
5 S Ribosomal RNA	C	18042	–
Uridine kinase	P	19174	2.7.1.48
Nucleosidemonohosphate kinase (Uridine monophosphate kinase)	C	19173	2.7.4.4
UDP glucose pyrophosphorylase	P	19175	2.7.7.9
Zonular pulverulent cataract (*Cae*)	P	11620	–
Chromosome 2			
Acid phosphatase-1 (red cell type)	C	17150	3.1.3.2
α-1-Antitrypsin (*Pi*)	P	10740	–
Galactose enzyme activator	P	13703	–
Hemoglobin (α- or β-)	P	14180–90	–
Interferon-1	P	14757	–
Immunoglobulin heavy chains (*Gm*)	P	14710–19	–
Isocitrate dehydrogenase-1	C	14770	1.1.1.42
Malate dehydrogenase-1	C	15420	1.1.1.37
Chromosome 3			
Aconitase (mitochondrial)	P	10085	4.2.1.3
Galactose-1-phosphate uridyltransferase	P	23040	2.7.7.12
Chromosome 4			
Hemoglobin (α- or β-)	P	14180–90	–
Phosphoglucomutase-2	P	17200	2.7.5.1
Chromosome 5			
Antiviral state repressor regulator	P	–	–
Diphtheria toxin sensitivity	C	12615	–
Hexosaminidase B	C	14265	3.2.1.30
Interferon-2	P	14758	–

(*continued*)

Chromosome 4 or 5			
FGAR amidotransferase (Ade B)	P	10255	–
Esterase activator	P	13325	–
Chromosome 6			
B-cell alloantigens	P	–	–
Chido blood group	C	11043	–
Complement component 2	P	12060	–
Complement component 8	P	–	–
C3 proactivator (properdin factor B)	C	13847	–
Glyoxalase I	C	13875	4.4.1.5
HLA-A	C	14280	–
HLA-B	C	14283	–
HLA-C	C	14284	–
HLA-D	C	15785	–
Immune response loci	P	14685	–
Malic enzyme-1	C	15425	1.1.1.40
Monkey RBC receptor	P	–	–
P blood group	P	11140	–
Pepsinogen	P	16970	–
Phosphoglucomutase-3	C	17210	2.7.5.1
Rodgers blood goup	P	–	–
Superoxide dismutase-2 (mitochondrial)	C	14746	1.15.1.1
Chromosome 7			
Colton blood group	P	11045	–
β-Glucuronidase	P	25322	3.2.1.31
Hageman factor (Factor XII)	P	23400	–
3-Hydroxyacyl-CoA dehydrogenase	P	13844	1.1.1.35
Kidd blood group	P	11100	–
Malate dehydrogenase-2 (mitochondrial)	C	15410	1.1.1.37
SV40-associated phenotypes	P		
-T antigen	P	18680	–
-transforming factors	P	19905	–
-transplantation antigen	P	19118	–
-V-antigen	P	–	–
-tumorigenic factors	P	–	–
-surface antigens	P	–	–
-viral DNA	P	–	–
Chromosome 8			
F VII factor regulator	P	–	–
Glutathione reductase	P	13820	1.6.5.2
Chromosome 9			
ABO blood group	C	11030	–
Aconitase (cytoplasmic)	P	10085	4.2.1.3
Adenylate kinase-1	C	10300	2.7.4.3
Adenylate kinase-3	P	10303	2.7.4.3
Nail-patella syndrome	C	16120	–

(*continued*)

TABLE III (*continued*)

Chromosome 10			
Adenosine kinase	C	10275	2.7.1.20
Glutamate-α-semialdehyde synthetase	P	13825	–
Glutamate-oxaloacetate transaminase (cytoplasmic)	C	13818	2.6.1.1
Hexokinase-1	C	14260	2.7.1.1
Pyrophosphatase (inorganic)	C	17903	3.6.1.1
Chromosome 11			
Acid phosphatase-2 (lysosomal)	C	20095	3.1.3.2
Esterase A_4	C	13322	3.1.1.2
Lactate dehydrogenase A	C	15000	1.1.1.27
Species antigen 1 (lethal antigen)	C	14873	–
Chromosome 12			
Citrate synthase (mitochondrial)	P	11895	4.1.3.7
Enolase-2	P	13136	4.2.1.11
Glyceraldehyde-3-phosphate dehydrogenase	P	13840	1.2.1.12
Lactate dehydrogenase B	C	15010	1.1.1.27
Serine hydroxymethyltransferase	P	13845	2.1.2.1
Triose phosphate isomerase	C	19045	5.3.1.1
Peptidase B	C	16990	3.4.11.-
Chromosome 13			
Esterase D	C	13328	3.1.1.1
Retinoblastoma-1	P	18020	–
Ribosomal RNA (18 S and 28 S)	C	18045	–
Chromosome 14			
GAR formyltransferase	P	–	2.1.2.2
Purine nucleoside phosphorylase	C	16405	2.4.2.1
(Ribosomal RNA (18 S and 28 S)	C	18045	–
Tryptophanyl-tRNA synthetase	C	19105	6.1.1.2
Chromosome 15			
Hexosaminidase A	C	27280	3.2.1.30
Hexosaminidase C	P	–	3.2.1.30
Isocitrate dehydrogenase (mitochondrial, NADP dependent)	P	14765	1.1.1.42
Mannose phosphate isomerase	C	15455	5.3.1.8
β_2-Microglobulin	C	10970	–
Pyruvate kinase (M2)	C	17905	2.7.1.40
Ribosomal RNA (18 S and 28 S)	C	18045	–
Chromosome 16			
Adenine phosphoribosyltransferase	C	10260	2.4.2.7
Antiviral state derepressor	P	–	–
α-Haptoglobin	C	14010	–
Interferon production	P	–	–
Lecithin:cholesterol acyltransferase	C	24590	2.3.1.43
Thymidine kinase, mitochondrial	P	18829	2.7.1.21

(*continued*)

Chromosome 17			
Adenovirus 12: Chromosome 17 modification site	C	10297	–
Galactokinase	C	23020	2.7.1.6
Thymidine kinase (cytoplasmic)	C	18830	2.7.1.21
Chromosome 18			
Chorionic gonadotropin	P	–	–
Peptidase A	C	16980	3.4.11.-
Chromosome 19			
Echo 11 virus receptor	P	12915	–
Glucose phosphate isomerase (phosphohexose isomerase)	C	17240	5.3.1.9
Peptidase D	P	17010	3.4.11-
Poliovirus receptor	C	17385	–
Chromosome 20			
Adenosine deaminase	C	10270	3.5.4.4
Desmosterol:cholesterol conversion enzyme	P	12565	–
Inosine triphosphatase	P	14753	3.6.1.3
Chromosome 21			
Glutathione peroxidase	P	–	1.11.1.9
Glycinamide ribonucleotide synthetase	P	13844	6.3.4.13
Interferon sensitivity (receptor)	C	10745	–
Ribosomal RNA (18 S and 28 S)	C	18045	–
Superoxide dismutase-1 (cytoplasmic)	C	–	1.15.1.1
Chromosome 22			
β-Galactosidase	P	–	3.2.1.23
Ribosomal RNA (18 S and 28 S)	C	18045	–
X Chromosome			
Dihydrotestosterone receptor	C	31370	–
α-Galactosidase	C	30150	3.2.1.22
Glucose-6-phosphate dehydrogenase	C	30590	1.1.1.49
Hypoxanthine phosphoribosyltransferase	C	30800	2.4.2.8
Ornithine transcarbamylase		–	2.1.3.3
Phosphoglycerate kinase	C	31180	2.7.2.3
Species antigen-X	C	31345	–
Tyrosine aminotransferase regulator	P	31435	–
Xg blood group	C	31470	–
Y chromosome			
H-Y antigen	P	–	–

[a] As decided at Baltimore Conference (Bergsma, 1976). "C" indicates the assignment has been *confirmed* by at least two independent methods or investigators. "P" indicates *provisional* assignment.

[b] McKusick number refers to the number in McKusick's catalogue of genetic traits in man (McKusick, 1975).

It will be of great interest to compare the quantitative intergene distances determined using this system with those derived from meiotic recombination distances. For example, the intergene distances determined from family studies recombination data are known for several loci on human chromosome 1. The order of these loci and a number of others on chromosome 1 have been determined using translocations and deletions. Thus, selective systems using, for example, the uridine kinase gene provisionally assigned to chromosome 1, or use of translocations between chromosome 1 and other chromosomes with selectable genes, could provide a direct comparison of meiotic recombination distance and distances as determined by the irradiated cell method.

B. Gene Mapping Using Ovarian Teratomas

It has recently been demonstrated that benign cystic teratomas of the ovary are derived from germ cell progenitors (Linder *et al.*, 1975). A number of teratomas have been analyzed and their isoenzyme and chromosome composition compared with those of the women from whom they were removed (Linder *et al.*, 1975; Ott *et al.*, 1976b). In all cases chromosome variants which were heterozygous in the host woman's karyotype were found to be homozygous in the teratomas. However, a number of isozymes which were heterozygous in the host were found to be heterozygous in the teratoma tissue as well. Two alternative explanations (Fig. 4) are that either second meiotic division was suppressed or that there was fusion of the ovum nucleus with the second polar

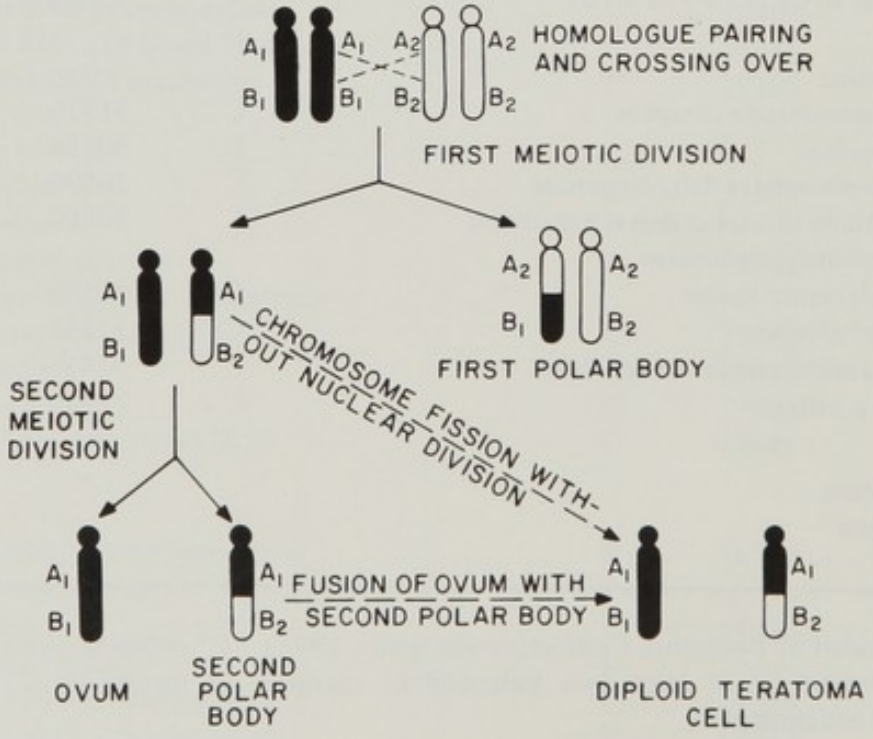

Fig. 4. Formation of ovarian teratomas (see text).

body (Gerald, 1975; Linder *et al.*, 1975). The resulting diploid cell then proliferated to form the teratoma.

Since the centromeres of each chromosome pair in the teratoma were derived from a single maternal host chromosome, chromosomal markers and genes located near the centromere are most likely to be homozygous in the teratoma, while genes located farther away from the centromere have a greater likelihood of being heterozygous due to crossing-over. The conditions for extracting genetic information from this fortuitous (for geneticists) occurrence are that:

1. Chromosomal variants be detectable in the maternal host and in the teratoma.
2. Phenotype (e.g., isozyme) variants be detectable in the maternal host and teratoma.
3. The phenotypes, to be useful, should have been previously assigned to specific chromosomes.

Let us assume that a maternal host is heterozygous for a centromeric fluorescence marker on chromosome 13, and that the host is also heterozygous for isozymes of esterase D, the gene for which has been assigned to chromosome 13. Examination of a teratoma derived from such a host would show homozygosity of the fluorescent centromere region, but could either be homozygous or heterozygous for the esterase D isozymes. By examining a number of such cases, one could get a quantitative measure of the distance from the centromere of the gene for esterase D, with the exactness of the localization increasing as the number of cases increased (Ott *et al.*, 1976).

A nice aspect of this system is that a single host–teratoma combination could supply information about several gene locations. If a large number of these host–teratoma pairs were collected and stored, they could provide an extremely valuable resource, particularly as our expertise in detecting chromosomal variants increases and as the number of isozymes and other polymorphic markers detectable in such cells increases. Since the teratomas contain tissue exhibiting various types of differentiated functions, the possibility exists to map differentiated functions. A severe limitation of the system is that the usefulness for mapping will decrease drastically as the distance from the centromere increases. Nevertheless, this method should provide much useful information in the near future.

C. Cot Analysis of Somatic Cell Hybrids

While *in situ* hybridization (Wimber and Steffensen, 1973; Price and Hirschhorn, 1975; also see Chapter 3 in this volume) is certainly useful for gene localization, the apparent lack of sensitivity of this method has limited its application. While Cot analysis using solution hybridization (Britten and Kohne, 1968) is a more sensitive method of detecting the presence of specific DNA sequences, it would not be possible to use this method for gene localization

on human cells alone, except where gene dosage existed (see Section II,D). Analysis of rodent–human somatic cell hybrid clonal lines, where each line contains specific human chromosomes, provides an alternate way of determining the assignment of specific sequences to particular human chromosomes.

Deisseroth *et al.* (1976; Deisseroth, 1976) have shown that cDNA made from hemoglobin mRNA can be used to detect α- and β-globin sequences in hybrid cells, and have further shown (Deisseroth *et al.*, 1976) that the α- and β-globin genes are on different chromosomes. It should be possible, therefore, to test the proposed assignment of the globin genes to human chromosomes 2 and 4 (Price *et al.*, 1972). It seems likely that cDNA made to any isolatable human mRNA, such as immunoglobulin mRNA, could be used for gene localization in hybrid cells, regardless of whether the particular gene is being expressed (i.e., transcribed) in the cells tested. Thus cDNA for immunoglobulin mRNA could be used to map the human immunoglobulin genes using cells which did not necessarily produce immunoglobulins. Cot analysis could also be used to localize genes to regions of chromosomes, using hybrid cells carrying the appropriate chromosome rearrangements.

A further use of Cot analysis in hybrid cells is to localize viral integration sites on human chromosomes. For example, Khoury and Croce (1975) have shown that rodent–human hybrids carrying human chromosome 7 carry the integrated genome of the SV40 virus, thus indicating that SV40 virus can integrate into at least chromosome 7 in man.

D. Chromosome-Mediated Gene Transfer

McBride and Ozer (1973) first clearly demonstrated the transfer into cells of functional genetic material utilizing isolated metaphase chromosomes. They showed that the Chinese hamster gene for HPRT could be transferred into $HPRT^-$ mouse cells via uptake into the mouse cells of isolated Chinese hamster metaphase chromosomes. Wullems *et al.* (1975, 1976a), Willecke and Ruddle (1975), and Burch and McBride (1975) have confirmed the earlier result; Willecke and Ruddle, using human chromosomes as the genetic donor, showed that the transferred piece was less than 1% of the total human genome, as judged by the absence in the transformed cells of other X-linked human enzymes. The apparent transfer of an intact human X chromosome has also been reported (Wullems *et al.*, 1976b).

Since the genes for thymidine kinase and galactokinase have been shown to be syntenic in man (Elsevier *et al.*, 1974), chimpanzee (Chen *et al.*, 1976; Orkwiszewski *et al.*, 1976), African green monkey (Orkwiszewski *et al.*, 1976), and the mouse (Kozak and Ruddle, 1976), co-transfer of these two genes was examined. A similar frequency of co-transfer of these two genes using both Chinese hamster (Burch and McBride, 1975; Ruddle and McBride, 1976) and human (Willecke *et al.*, 1976) donor chromosomes suggests an evolutionarily

conserved intergene distance, and demonstrates the potential application of this system in the determination of intergene distances. The main limitation to application of this system at present is the relatively small number of well-characterized selective systems.

ACKNOWLEDGMENTS

We wish to thank T.-S. Chan, S. Chen, C. Corsaro, B. Dorman J. Jensen, D. Leary, C. Partridge, M. Roberts and J. Tischfield for ideas and helpful discussions during the preparation of this manuscript, which would not have been completed without the help of M. Reger.

This work was supported by USPHS Grant GM 09966.

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5

Chromosomes and Chromatin Structure

G. F. BAHR

I. INTRODUCTION*

Two relatively recent developments have been primarily responsible for a quantum advance in our understanding of chromosomes and chromatin structure. The first was the discovery of a combination of preparatory steps that made it possible to observe whole chromosomes under the electron microscope (Gall, 1963). Of these steps, two were of particular importance: (1) the spreading of cellular organelles on a surface of water (Fernández-Morán, 1948; Kleinschmidt *et al.*, 1962), which had not previously been tried on chromosomes, and (2) drying by the critical-point method of Anderson (1951), which prevented the powerful surface tension of water from collapsing the delicate structure of chromosomes. The second development of major importance to chromosome research was the discovery that chromosomes could be made to display a characteristic banding pattern when stained with certain stains (Caspersson *et al.*, 1970a,b; Arrighi and Hsu, 1971; Arrighi *et al.*, 1971; Yunis *et al.*, 1971).

In this chapter an attempt will be made to describe the supramolecular structure of chromatin and its condensed form, the chromosome, as it is perceived in the laboratories of the author. No claim is made that this description is complete. Those areas of structure and function that are still poorly understood are, as is usual in such instances, filled with more or less reasonable hypotheses. Consolation for this situation may be found in the words of Einstein who said that "imagination is more important than knowledge."

II. COMPOSITION OF CHROMATIN

A. DNA

While participation of deoxyribonucleic acid in the organization of chromatin is axiomatic, a plethora of ideas have been put forth in the past as to how this DNA may participate in the structure of a chromosome. Through influence from dipteran cytogenetics, the idea persisted that DNA in mammalian chromosomes is multistranded. From this concept followed the notion that two or more subchromatids constitute one chromatid, and this was indeed helpful in explaining some experimental results, such as isochromatid labeling.

Taylor *et al.* (1957) provided the first strong evidence that each eukaryotic chromatid is comprised of one, and only one, very long duplex molecule of DNA. This idea and the underlying experimental facts supporting it have gained

*The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

wide acceptance in recent years (see Prescott, 1970; Laird, 1971; Comings and Okada, 1969). Experiments with sister chromatid exchange (Latt, 1974a,b) and other rearrangements of chromosomal parts are most easily understood when the chromatid is considered to be comprised of one single, uninterrupted long duplex molecule (the unineme or, to be more correct linguistically, the mononeme).

As a basis for estimations of the length of DNA molecule(s) in chromosomes or chromatin, we will consider a recent report by Wray (1972). This author reported on the molecular weight of DNA isolated from chromosomes by mild methods. About 400–500 daltons $\times 10^6$ can be gleaned from their sedimentation profile as the maximum mass of all experimentally found DNA molecules. Taking MacHattie and collaborators' (1965) value of 1.92×10^6 daltons/μm, we calculate roughly 240 μm for this longest piece of DNA. Taking 4.32% as the percentage of DNA that is incorporated in human chromosome No. 1 (Mendelsohn *et al.*, 1973), a total molecular length of 4.7 cm is calculated for one chromatid where the mass of 1 μm of DNA is 3.14 (MacHattie and Thomas, 1964) or 3.15×10^{-18} gm/μm (Leighton and Rubenstein, 1969). Others have reported 22,000 μm as the longest piece of DNA observed (Sasaki and Norman, 1966). The mass values given refer to the sodium salt of DNA in the B configuration, the predominant configuration in unfixed nuclei (Langridge *et al.*, 1960).* Why does such comparatively short chromosomal DNA frequently appear? (Adenovirus type 2 contains 10.9 μm duplex; Doerfler and Kleinschmidt, 1970.) One might suspect breakage in spite of gentle isolation procedures. The hypothesis that protein linkers, e.g., amino acids, are interspersed between long DNA molecules to facilitate uncoiling for synthesis has not found experimental support.

In this chapter only brief allusion to the molecular heterogeneity of mammalian DNA will be made. All mammals possess repeated nucleotide sequences in their DNA, though the number and complexity of repeated copies varies widely (Britten and Davidson, 1971). A short interspersion pattern has been described for sea urchins and *Xenopus leavis*. A large portion of the genome consists of short sequences of approximately 300 base pairs of moderately repetitive DNA adjacent to segments about 1000 base pairs long, constituted of nonrepetitive DNA. This is taken to mean that pieces of transcribed genome are separated by 300 base pairs of nontranscribed DNA. Other, much longer, repetitive molecules have been reported, the rule being that the longer the repetitive sequence, the fewer the molecules to be found. Structural genes are variously assumed to occupy 2–50% of the available DNA. Whatever the value is,

*Stubblefield and Wray (1974) reported a density of 1.36 gm/cm^3 for dry chromosomes (determined by centrifugation). This is in excellent agreement with our electron microscopic determinations (DuPraw and Bahr, 1969).

it appears that a large portion of the nuclear DNA content functions in a role supportive of the genome. This role must be essential, since nonfunctional DNA would have long been deleted by evolutionary pressures. Taylor and Hozier (1976) report on a 4-μm-long replication unit in CHO as revealed by autoradiography. A similar replication length would imply (2,200,000 μm:4 μm) that there are about 550,000 initiation sites in the diploid nucleus.

It is now certain that a given mammalian species possesses a constant amount of DNA (for a recent review, see Kraemer *et al.*, 1972). The human diploid chromosomal complement contains, according to our own determinations (M. Cimino, personal communication), 6.9×10^{-12} gm of DNA (7.0×10^{-12} gm according to Ohno, 1967). Dividing this value by the 3.14×10^{-18} gm/μm of DNA double helix cited, we calculate 2.2 meters (2,200,000 μm) of DNA per human diploid nucleus.

B. Nuclear Proteins

The eukaryotic chromosome is composed of DNA associated with an approximately equal amount of histone protein and a variable but relatively large amount of nonhistone protein. Taking a normal human lymphocyte as an example of a human diploid cell in G_1, the following values pertain: total DNA content 6.90×10^{-12} gm, total dry mass (mode) of 45.0×10^{-12} gm (Golomb and Bahr, 1974a). If a DNA content of 15% is calculated for the lymphocyte nucleus and an equal 15% for histone protein, one must come to the conclusion

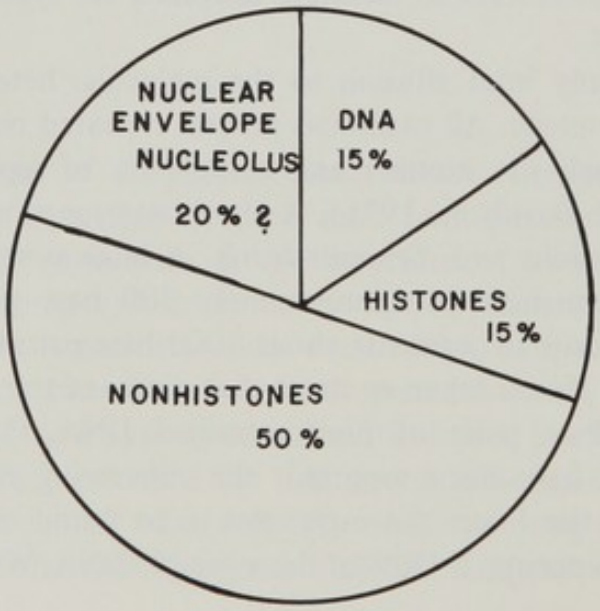


Fig. 1. The composition of the nucleus is given in percentages. The fraction of nuclear envelope and nucleolus is calculated from the thickness of the envelope. The mass of a nucleolus is taken as 0.4×10^{-12} gm from interference microscope measurements (Stenram, 1957).

that the remainder 70% represents nonhistone protein, the nuclear envelope, and the nucleolus and other chromatin-associated RNA (Fig. 1). No data are available for the proportionate mass of nuclear envelope and nucleolus of this relatively inactive cell. It is estimated at 20%. Whatever mass is assigned to these two structures, the fact remains that nonhistone proteins constitute a major fraction of the nuclear content.

There is evidence (Golomb and Bahr, 1974a) that nearly all of the content of a diploid nucleus is, on a weight basis, found doubled at the tetraploid state of metaphase and incorporated into chromosomes. From this it follows that practically all of the nuclear content is incorporated in either chromatin or the nucleolus.

The DNA content of metaphase chromosomes has been reported to be slightly higher than for interphase chromatin (Huberman and Attardi, 1966). If metaphase chromatin contains 25% DNA and an equal percent of histones, one would still have to consider about 50% nonhistone protein. All of this leads to the repeated argument that whole chromatin, of interphase as well as of metaphase, contains *at least 50% nonhistone protein incorporated into chromatin fibrils*.

While the role of the nonhistone proteins in chromatin organization remains largely unknown, progress has been made toward understanding the histones. For the most part this has related to the chemical composition of the histones and their structural organization into chromatin. The functional significance of the various types of histone proteins remains a subject for hypotheses.

In the past decade it has been possible to isolate and identify essentially five distinct classes of histone proteins. The history of this development is reflected in the original nomenclature for histone fractions (Table I). According to amino acid composition, three major groups are recognized: lysine-rich, slightly lysine-rich, and arginine-rich. Although the amino acid sequence for four histones has been established (Elgin *et al.*, 1971), the possibility of minor heterogeneities remains, especially for the lysine-rich histone H1.

Lysine-rich histone H1 occurs as an octamer and is very eaily removed from chromatin. We shall have much more to say about the role of H1 in chromosome banding.

There are two fractions of slightly lysine-rich histones, H2A and H2B, and two fractions of arginine-rich histones, H3 and H4, all of which have been conserved during evolution and across such diverse species as pea and cow (Bonner *et al.*, 1968; Elgin *et al.*, 1971) with only minor changes in amino acid composition. Histones H3 and H4 appear to be the most highly conserved proteins known (Lewin, 1974, 1975a). Lewin writes "such striking suppression of variation suggests that the entire sequence of each of these histones is involved in its function and that this function may be virtually identical in all eukaryotes. A function common to such different species presumably must be

TABLE I
Nomenclature for Histone Protein Fractions

Histone	Older codes[a]		New code[b]
Lysine-rich	f1	Ib	H1
	(f2c	V	H5)[c]
Slightly lysine-rich	f2a2	IIb1	H2A
	f2b	IIb2	H2B
Arginine-rich	f3	III	H3
	f2a1	IV	H4

[a]Reflecting the development of understanding of histones.
[b]Bradbury (1975).
[c]In birds.

structural" and therefore a common feature of the molecular organization of all eukaryotic chromatin (Lewin, 1975b).

From observations that the slightly lysine-rich histones H2A and H2B form oligomers, the arginine-rich histones H3 and H4 aggregate to form a tetramer ($H3_2$–$H4_2$), and that oligomers and tetramers in the presence of DNA form nucleosomes, Kornberg (1974) suggested that a nucleosome of distinct composition is formed, involving 200 base pairs of DNA in a basic repeating unit. Other experiments are consonant with this general model (Hewish and Burgoyne, 1973; Clark and Felsenfeld, 1974; Sahasrabuddhe and Van Holde, 1974; Axel *et al.*, 1974; Pardon *et al.*, 1975; Olins and Olins, 1974; Langmore and Wooley, 1975; Shaw *et al.*, 1974; Slayter *et al.*, 1972; Oudet *et al.*, 1975; Lacy and Axel, 1975).

In spite of some discrepancy as to the shape and size of the nucleosome, the number of base pairs tightly associated with it, and the length of internucleosomal DNA, it is clear that the nucleosome is a basic unit of eukaryotic chromatin. Its approximate dimensions are 70–125 Å of disk or spherical shape, with 140–200 base pairs incorporated in it. The internucleosomal piece of DNA is variously indicated as 150–350 Å in length. Langmore and Wooley (1975) found a bimodal distribution in the length of the connecting DNA duplex, with modes at 200 Å and 350 Å.

Recent experiments of Gottesfeld *et al.* (1975) indicate that only the inactive portions of DNA feature nucleosomes. One of the current concepts sees histone H1 in the role of inducing and stabilizing the supercoil of nucleosomes and internucleosomal DNA and their packing into the chromatin fiber. In the absence of H1, the nucleosomes are revealed as beads on a string of naked DNA in the electron microscope (Oudet *et al.*, 1975).

Only when histones can be crystallized will it be clear whether the homogeneous fractions—by biochemical standards—indeed represent unique proteins or

whether small but significant variations determine conformation and site of attachment. Nucleosome-like particles in which the histone H3 is missing have been described for yeast by Lohr and Van Holde (1975). It is unknown whether this variant occurs in mammalian chromatin. The *in vitro* reassembly onto DNA and the simple repetitive nature of nucleosomes speak against binding to specific DNA sequences.

Among the nonhistone proteins, a large fraction consisting of contractile and structure proteins has recently been discovered. Douvas *et al.* (1975) reported that myosin, actin, and tubulin constitute 38% of the nonhistone fraction. This implies that the various movements of chromatin, such as migration toward the nuclear envelope or toward the nucleolus, the condensation of chromosomes, and their movement in mitosis and meiosis, are somehow dependent on the presence of contractile proteins. Many of the known enzymes of the nuclear genetic machinery, as well as many that have not yet been discovered, will most likely be found in the nonhistone fraction. A very comprehensive review of chromosomal proteins was published by Johnson *et al.* (1974).

III. FIBROUS NATURE OF EUKARYOTIC CHROMATIN

The nonnucleolar content of the nucleus is, for the most part, organized into a fibrous structure that has been given the name chromatin fiber. At this time nothing can be said about the number of chromatin fibers in a human diploid nucleus, but, as will be discussed later, this number is relatively small—at any rate not exceeding the number of chromatids, which is 92.

Some general properties of the fiber are well understood.

A. Fiber Diameter

Human chromatin fibrils have an average diameter of 199 Å ± 15 Å, in the following discussion called the 200 Å fiber. The measured diameter has been found to range from 120 to 250 Å for unarrested metaphases and from 30 to 250 Å for interphase nuclei (Bahr and Golomb, 1974).

A wealth of measurements of chromatin fibers, from both whole-mounted and sectioned nuclei and chromosomes, are available from the literature. In agreement with our observations, the thinnest fiber encountered by Watanabe and Tanaka (1972) measured 30 Å. They also found fibers from human lymphocytes in the range of 200–300 Å. Ris (1967) and Ris and Chandler (1963) reported fiber diameters ranging from 20 to 250 Å (the latter for a pair of twisted 100 Å fibers). Maximum fiber diameters have been variously reported as: 500 Å (Stevens, 1967), 250 Å (Schwarzacher and Schnedl, 1969), 280 Å (Wolfe, 1965a,b), 205 Å (Heumann, 1974), 250 Å (Lampert and Lampert, 1970;

Lampert, 1971a,b), and 191 Å (Zirkin and Kim, 1972). Many more investigators have mentioned fiber diameter in passing.

There has been indication of some degree of interspecies variation in fiber diameter. DuPraw (1965) reported 230 Å fibers in the honey bee. Isolated nucleoprotein from calf thymus was found to contain fibers with a diameter of 150 Å (Littau *et al.*, 1964).

It should be noted that among the above cited examples, the investigators who used glutaraldehyde-osmium tetroxide fixation on whole cells were closest to the 200 Å value found by us (Zirkin and Kim, 1972; Lampert, 1971a,b; Lampert and Lampert, 1970; Heumann, 1974).

A series of articles by H. G. Davies and collaborators published from 1973 to 1976 in the Journal of Cell Science (Great Britain) came to the attention of the author. In thin sections of goldfish nuclei fixed in glutaraldehyde, followed by osmium tetroxide a diameter of 174 Å was found. Our data on fixed chromatin fibers are in fair agreement with Dr. Davies' results. The center to center distance of neighboring fibers was found to be a rather constant 280Å ∓ 40Å, implying that the 174 Å cores are separated by about 100 Å space. Davies considers this space to be an integral part of the chromatin fiber. It is also possible that the irregularities of shape of the fiber are in this order of magnitude, preventing a closer side-by-side packing.

As is obvious from the preceding discussion, we consider 200 Å as the normal diameter for human chromatin at interphase and metaphase. The question as to how such varied measurements could be obtained by different researchers deserves some attention. Measured diameters in excess of 200 Å may be the result of the following phenomenon. In 1974, Bahr and Golomb investigated the effect of colchicine on chromosomal fibers and found that the common cytologic observation of highly condensed chromosomes after long exposure to this mitotic poison was mirrored at the fiber level by increased fiber diameters. Thicknesses of 500 Å and more were not unusual for these fibers (Bahr, 1970). In consequence, every fiber measurement made after the use of colchicine or other mitotic poison is suspect of this effect. A simple, though not proven, explanatory hypothesis for the colchicine effect envisages that excess calcium in the area of the nucleus is not chelated because no spindle fibers are formed. This excess calcium would act on the recently discovered contractile proteins in chromatin in a way comparable to its role in the contraction of muscle. A telescopic shortening and thickening of the fiber seems to proceed without adding to the mass of the chromosome itself (Golomb and Bahr, 1974a). Turning to decreased fiber diameter, we found our review of the literature to reveal that the more manipulation is used in obtaining chromatin, the thinner the fibers will be. Physical forces used in isolating nuclei by disruption of cells tend to stretch chromatin. We learned from chromatin observed in the electron

microscope that the mechanical forces of surface spreading can easily lead to stretched fibers, which invariably have lower diameters than unstretched fibers. The addition of chemicals in the form of buffers or chelating agents also tends to diminish the thickness of fibers through extraction. The latter point was established in our laboratories when the loss of proteins from isolated chromosomes was assayed (Retief, 1976). Water alone can extract appreciable amounts of protein, mostly the nonhistones, over a period of time. The addition of salt, as in buffers, accelerates this extraction process and affects the histones.

In *thin section* studies of fiber diameter, the observer is confounded by fibers leaving the plane of section at a flat angle and being cut at a grazing angle. This leads to images of fibers with apparently reduced diameter. The otherwise interesting work of Heumann (1974) is an example of this stereological problem. We remeasured representative chromatin fibers from his published electron micrographs at the indicated magnification and arrived at 200 Å ± 25 Å. Measuring all profiles, including those that were obviously cut and stretched as well as fully preserved ones, we arrived at the erroneously low mean value of 125 Å ± 27 Å.

The diameter of chromosomal *fibers spread on water* is subject to a number of variables; the major ones are additive to the water, such as extraction by salts or fixatives. Our experiments rendered the values collated in Table II (see also the comprehensive review by Solari, 1974).

While the control is close to our previously published value (Bahr and Golomb, 1974) of 199 Å, we find that both formalin and glutaraldehyde induce a 15% shrinkage in diameter. From this, we infer that a shrinkage in the longitudinal direction has also occurred. This is a process well known to electron microscopists: whenever an aldehydic fixative is used, there is a strong tendency of chromatin to "marginate," i.e., the chromatin contracts onto the inner nuclear membrane. This margination is not seen when osmium tetroxide alone is used as the fixative on normal cells.

Both uranyl acetate and osmium tetroxide reduce the diameter of isolated

TABLE II
Diameters of Chromatin Fibers after Various Treatments

	Å	SD	n
Control	197	43	157
Formalin, 10%, pH 6.8, unbuffered	165	40	92
Glutaraldehyde, 2%[a]	166	38	89
1% uranyl acetate[a]	171	45	27
OsO_4[a]	142	47	72

fibrils when they are directly exposed for 30 minutes. This reduction in diameter by either glutaraldehyde or osmium tetroxide does not take place when whole cells or tissue are fixed, as was mentioned before.

There is little evidence from studies of isolated chromatin in which mean fiber diameters of 100 Å or less have been reported that these fibers possess the same composition and properties as nuclear chromatin *in situ* or that significant quantities of proteins have not been lost in the preparation procedure. This is especially true when salt solutions and physical forces have been involved in the isolation process (Bram and Ris, 1971; Pardon and Wilkins, 1972; Dusenberry and Uretz, 1972). We ourselves added to the confusion regarding chromatin fiber diameters when we reported values from 50 to 500 Å (DuPraw and Bahr, 1969; Bahr, 1970) before the effects of mitotic poisons were discovered.

B. Fiber Mass

It is possible to evaluate contrast in an electron micrograph of a whole-mounted object or its parts to determine their dry mass. The procedure is briefly outlined in Section VII. Such dry mass measurements are independent of the specimen's shape, chemical composition, or internal organization. A detailed description is given in a review by Bahr (1975).

Selecting only relaxed fibers, i.e., those that have not been drawn fully straight, we determined a mean mass of 5.95×10^{-16} gm/μm fiber ± 29% (Golomb and Bahr, 1974a). This value supersedes all other measurements on chromosomes previously published by this laboratory. Earlier reports erred due to colchicine effect (*vide supra*). The dry mass of the contents of an average lymphocyte nucleus has been determined to be 45×10^{-12} gm. When the statements at the beginning of this chapter regarding nuclear composition and the almost complete incorporation of nuclear material into fibers are kept in mind, one must realize that the 45 picograms are chromatin fibers. Dividing the nuclear content of fibers by the mass of 1 μm of fiber (5.95×10^{-16} gm/μm), we arrive at 76,000 μm or 7.6 cm chromatin fiber per nucleus.

Doing more arithmetic, we calculate a volume of 23.9×10^{-12} cm^3 for a 200 Å cylinder 7.6 cm in length. Considering a nucleus of 120×10^{-12} cm^3, which reflects the average-size nucleus in a lymphocyte with 28% dry mass, one finds a surprisingly low percentage (20%) of the nuclear cavity occupied by chromatin fibers. Of course, the nucleolus has to be added, but on the whole, one can now understand how easily a shifting of the content can produce the impression of large "empty" spaces. This is particularly apparent when the chromatin has contracted onto the nuclear membrane. Because such contraction cannot fully compress chromatin, we are seeing about 40% of random nuclear cross sections occupied by chromatin.

C. Configuration of the Chromatin Fibril

Knotty, kinky, irregular, and bumpy are some of the adjectives that have been used to describe the appearance of a chromatin fibril. Nothing can better describe it than an electron micrograph (Fig. 2). Ris (1966) and Bram and Ris (1971) depicted knobs and side arms as integral parts of a 100 Å fibril. These features have to be considered in any general model of chromatin.

The uniquely irregular fiber appears nevertheless to have an overall regularity of diameter and shape. To put this to a test, various negative and positive electron micrographs were placed in a laser diffractometer. For comparative purposes, wire models and photographs of India-ink drawings resembling various areas of a chromosome were diffracted. As could be expected, a wire model or neat ink drawing produced broad diffraction rings; but as the evenness of the ink line deteriorated or several thicknesses of wire were used, all diffraction patterns weakened and finally disappeared. This is only to say that chromosomes, *critical-point-dried and whole-mounted, do not possess structural regularities in*

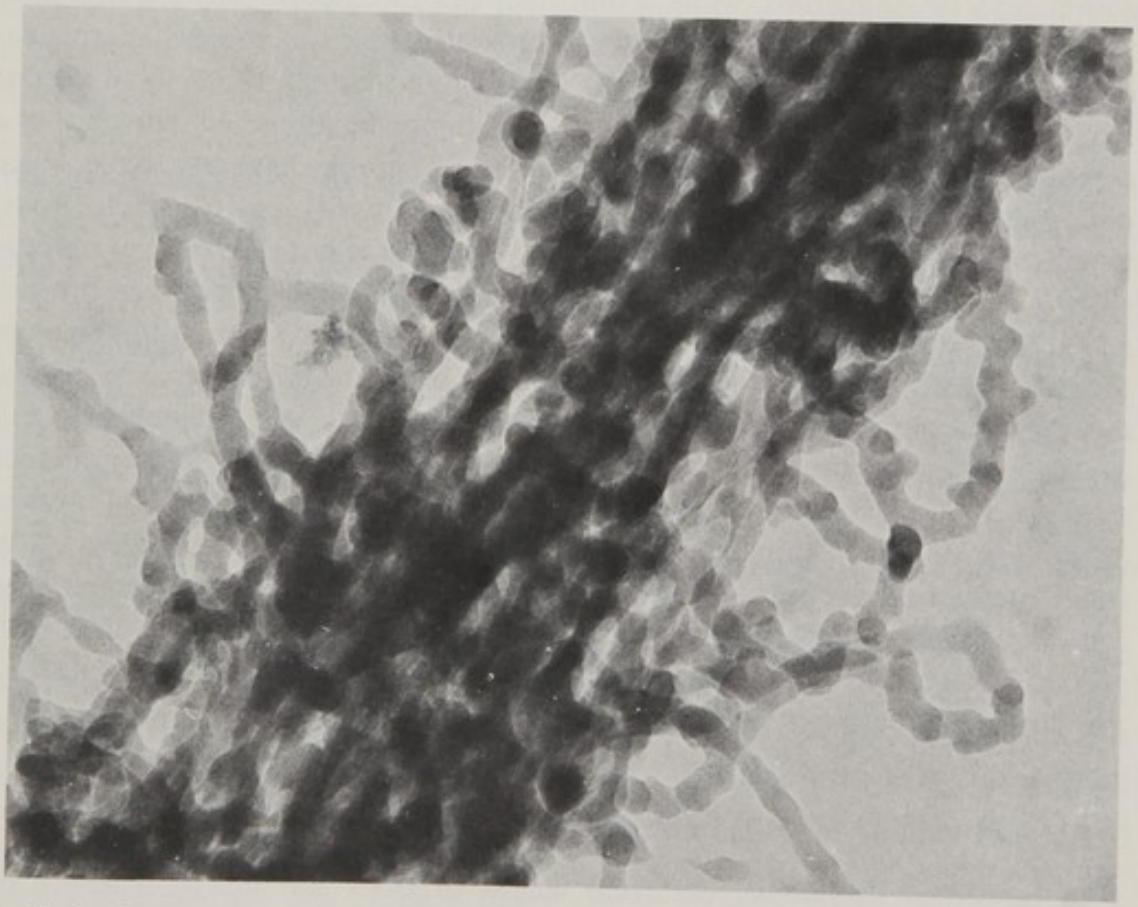


Fig. 2. Typical fiber configurations are seen close to an interchromomeric section of a chromosome. Note the partly smooth, partly irregular outline of the 200 Å fiber.

any part that suffice to give an optical diffraction pattern. Irregularity as a feature of chromatin observed under the electron microscope is thus confirmed by optical means.

Another sign of irregularity in chromatin fibrils is evident when fibrils of interphase nuclei or of chromosomes are stretched in the process of spreading or attaching themselves to grids for electron microscopy. One sees portions of a fiber with a regular diameter alternate with attenuated portions down to the very thinnest fibers detectable in unstained preparations (around 30 Å). There is no apparent pattern in the manner in which thick and thin portions alternate. A very long, thin segment of a fiber may be interrupted in the middle by a knoblike thick segment. Likewise a thin portion may abruptly be interspersed between two thick segments. This situation was illustrated in part by DuPraw and Bahr (1969) when we defined thin fibers as type A and a normal fiber as type B. Our Fig. 10 illustrates the considerable spread of diameters for the thinner type A fiber and the continuous range of diameters of both types of fibers displayed. The normal fiber has a coefficient of variation of 15%, while thin fibers vary up to ±50% of their mean. A normal fiber is structurally variable within tens of Ångstroms, while a thin fiber can be smooth for long stretches.

In high resolution cross sections of chromatin fibers, a somewhat irregular ring can sometimes be seen, which led Lampert and Lampert (1970) to suggest that chromatin fibers may be hollow. Our own investigations have shown that a cross section is almost never truly circular or oval but may have any shape. More importantly, a cross section seldom exhibits an even density to electrons. Usually one side or corner has less contrast, and this is not the result of oblique cutting, as stereopairs have shown. We conclude that uneven contrast profiles must reflect either structural features or local differences in reactivity to osmium tetroxide (Bahr, 1954). When the irregularity of chromatin fibers is viewed in the light of possible regularity at the molecular level, one cannot help but wonder whether the preparative techniques used for electron microscopy induce artifacts that mask or collapse regular structures. The methods of optical diffraction have been employed as an objective means to detect any regularity of fiber diameter in electron micrographs. This approach, which would generate an optical diffraction pattern on the basis of an average regularity in diameter (this printed page contains sufficient regularity in the typeface to generate a distinctive diffraction pattern), has failed to detect any regularity.

D. Ratio of Lengths of DNA to Chromatin Fiber

In preceding paragraphs the total length of DNA and the total length of chromatin fiber in a human diploid nucleus have been calculated. If these two are compared, we find the ratio DNA:chromatin fiber = 220 cm:7.6 cm or

28.94:1. In other words, there are approximately 29 lengths of DNA in every length of chromatin fiber.

The simplest way to accommodate so much DNA in a 200 Å fiber appears to be a supercoil, and several models of such an organization have been proposed (Bahr, 1970). Such models consider only two global quantities, DNA and fiber length. How does the new insight into the nucleosomal substructure of chromatin agree with the observation that chromatin is organized into a fibrous structure with an average diameter of 200 Å? We can approach this question with a modest experiment, namely, gluing spheres, having a diameter of 9 mm, at intervals of 20 mm to a jute cord 2 mm thick. Those familiar with published measurements on nucleosomes will recognize that a very simple model is being constructed. If a flexible plastic tube or hose with an inner diameter of 20 mm is placed on end on a flat surface, one can easily pack 15 spheres, attached and connected with cord, into a 40 mm length of tube. The spheres are mostly layered in sets of three to a plane approximately perpendicular to the axis of the tube. Assuming, as do Pardon *et al.* (1975), that 140 base pairs are integrated into a nucleosome and 60 base pairs constitute the internucleosomal DNA, we have 10,200 Å of DNA double helix in 400 Å (40 μm) fiber length. This represents a packing ratio of 25.5, in fair agreement with the previously estimated packing ratio of 28.3. Such a simple model leaves more than about half of the volume of the tube free for the missing histone H1 and nonhistone proteins. We should not fail to emphasize that there was much irregularity in the position of the spheres and the course of the thread (DNA molecule). In fact, the outside of the tube resembled the familiar irregular shape of a chromatin fibril.

At present the nature of nucleosomes is under intensive investigation. Noll (1974), Shaw *et al.* (1974), and Sollner-Webb and Felsenfeld (1975) estimate 600–680 Å as the length of DNA associated with a nucleosome. If, in the above model, 680 Å (or 200 base pairs) is used in the calculations instead of 140 base pairs, the packing ratio for DNA in a fiber would exceed 33. The size and shape of the nucleosome, the length of internucleosomal DNA, and the degree of mutual penetration of nucleosomes in tight packing represent other variables that affect the packing ratio. The concept of nucleosomes being held together and perhaps controlled by specific proteins, among them histone H1, nonhistones, and contractile proteins, is attractive for several reasons. First, the observed thickening of chromatin fibrils upon exposure to a mitotic poison is much easier to understand with nucleosomes than with a relatively rigid coiled coil configuration. Second, it explains images of 100 Å fibers emerging from 200 Å fibers (100 is the approximate diameter of nucleosomes). Further, the observed intermediate and thinner fibers can be imagined, since DNA stretches devoid of nucleosomes must be seen as part of the picture.

Why is the packing ratio independent of proteins picked up by chromatin

fibers in the preparation procedure? In calculating a packing ratio, one divides the length of the chromatin fibrils(s) L_F into the length of DNA double helix, L_{DNA} [Eq. (1)].

$$L_{DNA}/L_F \tag{1}$$

L_F is calculated simply by dividing the mass of the chromosome M_{Chr} by the average mass of 1 μm of chromatin fibril $\bar{M}_F/\mu m$ [Eq. (2)].

$$M_{Chr}/M_F\ \mu m = L_F\ \mu m \tag{2}$$

L_{DNA} is obtained in an analogous way by dividing the mass of 1 μm of DNA = 3.15×10^{-18} gm/μm into the mass of DNA of the chromosome or nucleus in question [Eq. (3)].

$$\frac{M_{DNA}}{6.24 \times 10^{-18}\ \text{gm}}\ \mu m = L_{DNA}\ \mu m \tag{3}$$

Inserting Eqs. (2) and (3) in (1), we obtain the ratio $L_{DNA}\ \mu m/L_F\ \mu m$.

Let us assume that the fibrils of a chromosome or nucleus adsorb proteins from the cytoplasm or the growth medium while being exposed to hypotonic media or disrupted on the water surface of the spreading trough. Let us further assume that the uptake increases the total mass by 100% and that this adsorption is fairly random, i.e., all fibrils are uniformly involved. The fiber mass per micrometer will be double, $\bar{M}_F$ will be $2\bar{M}_F$, and the increase in total mass will be $2M_{Chr}$. Inserting these into Eq. (2), we find that the increase cancels out. We can conclude that *any* amount of protein adsorbed by nucleus or chromosome will *not* influence the packing ratio. The fiber must telescope onto itself or stretch in order to change this ratio. We know that colchicine may produce telescoping.

IV. ARRANGEMENT OF CHROMATIN FIBERS IN CHROMOSOMES

Semiconservative replication of DNA (Taylor *et al.*, 1957) dictates that when a DNA strand replicates, two–in every respect identical–DNA daughter strands are synthesized. Each one is associated with protein, which is in all likelihood also identical for both strands. It is clear that out of one chromatin fiber, two identical chromatin fibers are produced. These are present in extended form in the interphase nucleus. When the cell passes through G_2, the two fibers condense to eventually constitute one-half of a chromosome, maintaining their twin character as they fold each into sister chromatids. Each individual chromosome is folded into a unique configuration at metaphase. This folding is precisely controlled and is, for a given species, repeated with great accuracy from generation to generation. Genetic variants, as expressed in chromosome shape (homo-

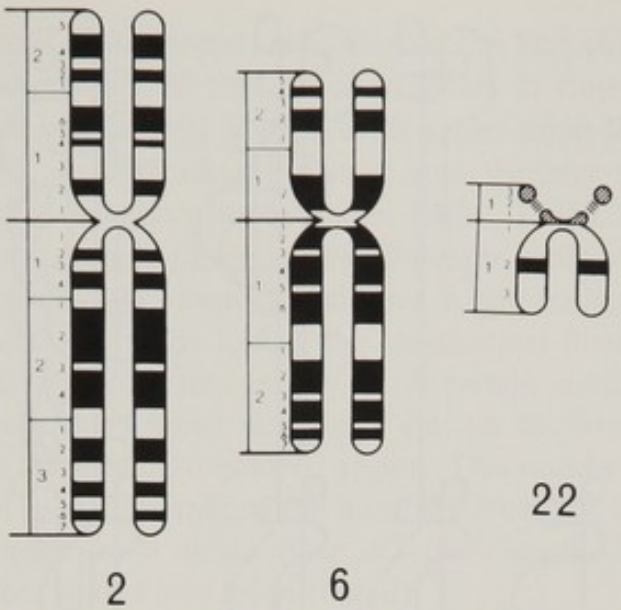


Fig. 3. Three chromosomes reproduced with permission from the Paris Conference (1971), Publication of the National Foundation. The principle of symmetry is implicitly incorporated in such representations.

logue variability), are reproduced with the same exactitude. The precision of this process extends beyond the determination of the shape of the chromatids, as seen in the microscope, to the molecular level, where the position of every molecule and the conformation of every molecule is determined. Early in the folding process the chromatids rotate 90 degrees toward one another. In this position they constitute congruent mirror images.

An example of this symmetry are chromosomes from the idiogram of the Paris Conference (1972) (Fig. 3). An imaginary plane transsecting the middle of the centromere in the direction of the long axis of one of the chromosomes would be the plane of symmetry for every structure and every molecule of the chromosome. This view of a chromosome is not presented in detail without good reason. When it comes to discussion of the structural basis for chromatid exchanges, explanation is heavily based on the concept of symmetry.

A. Folded-Fiber Model*

Within a single chromatid we believe that the fiber is folded in the manner illustrated in Fig. 4. The principle of the fiber folding into a chromomere, then

*The current model developed from original ideas of a folded-fiber model by DuPraw (1966). The model was refined, based on quantitative considerations of chromatin fibers and their arrangement in a chromosome (DuPraw and Bahr, 1969; Lampert *et al.*, 1969) and further modified according to continuing quantitative and qualitative findings (Bahr, 1975). In addition to those previously named, the following people were involved in quantitatively assessing eukaryotic chromatin: M. Cimino, H. Cyr, W. Engler, H. Golomb, R. Green, H. Herr, P. Larsen, U. Mikel, E. Pihl, A. Retief, and R. Ruechel.

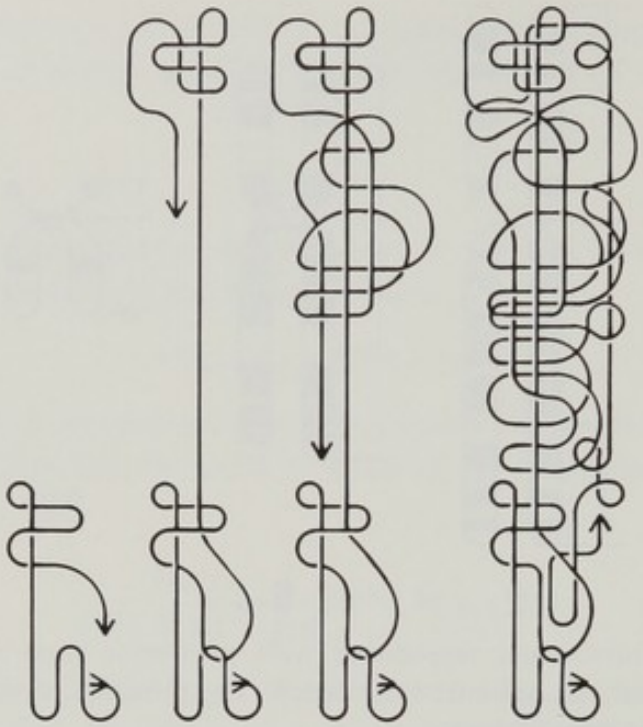


Fig. 4. A schematic and very much simplified pattern of the folding of a long chromatin fiber into a chromatid. Arrow indicates the transcriptional polarity of the fiber. Reprinted from G. F. Bahr (1975). *Fed. Proc.* **34**, 2209–2217.

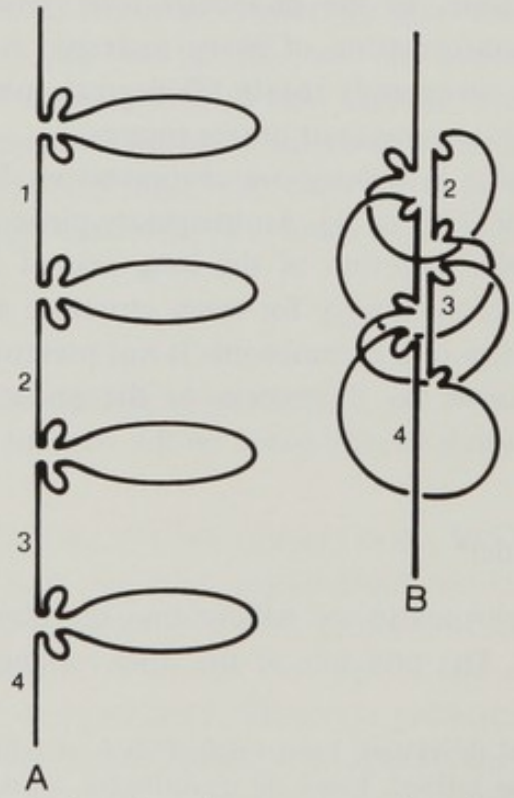


Fig. 5. This figure is intended to show how a mammalian chromosome structure may have originated from a lampbrush chromosome. In A, the principal folding pattern of a lampbrush chromosome is depicted. In B, sections of the central filament have been sliding past each other. Loops are now folded in a manner comparable to Fig 4.

proceeding to the next chromomere, returning to the first chromomere, and so on, is based on observations of the course of fibers in stereopairs. It remains debatable whether a longitudinal fiber extends across immediately neighboring chromomeres to link up with distant centromeres. We have argued that this is the case at least after a pericentric inversion (Bahr, 1975). The idea may be entertained that longitudinal fibers are evolutionary analogues to the central filament of lampbrush chromosomes and that a shortening of a lampbrush configuration is accomplished by sliding the longitudinal fibers past each other in a fashion tentatively illustrated in Fig. 5. A certain similarity to Fig. 4 is apparent. It has been determined that there are, on the average, 8–15 longitudinal elements in the interchromomeric region. This number has been derived by counting, as well as by determining the mass of a length of fibers and dividing this mass by the mass for a single fiber of equal length. Chromomeres are accumulations of fiber folded into loops (Fig. 6).

One may ask if there is a limit to the extension of a chromosome or a maximum contraction–condensation. Both points must be answered in the

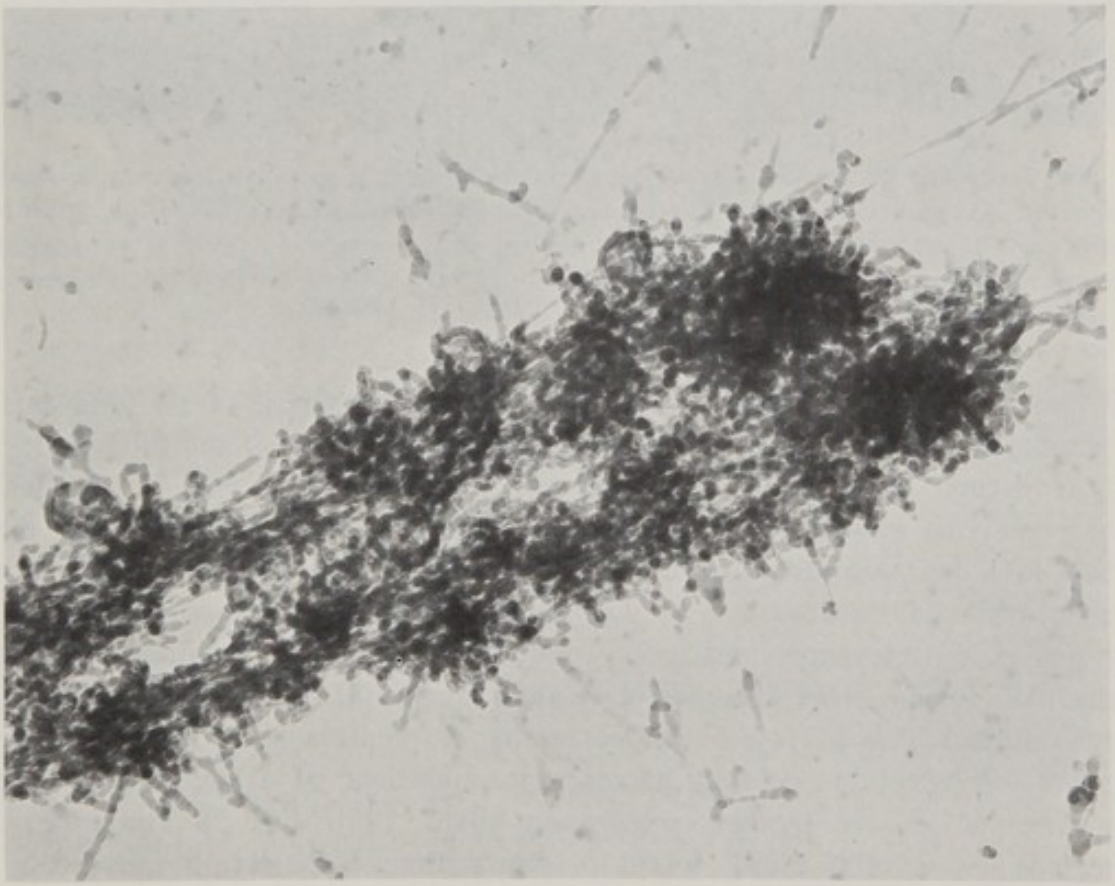


Fig. 6. This electron micrograph of an unfixed, critical-point-dried specimen shows the chromomeres as local accumulations of looping fibers and illustrates the presence of longitudinal fibers throughout the chromatid.

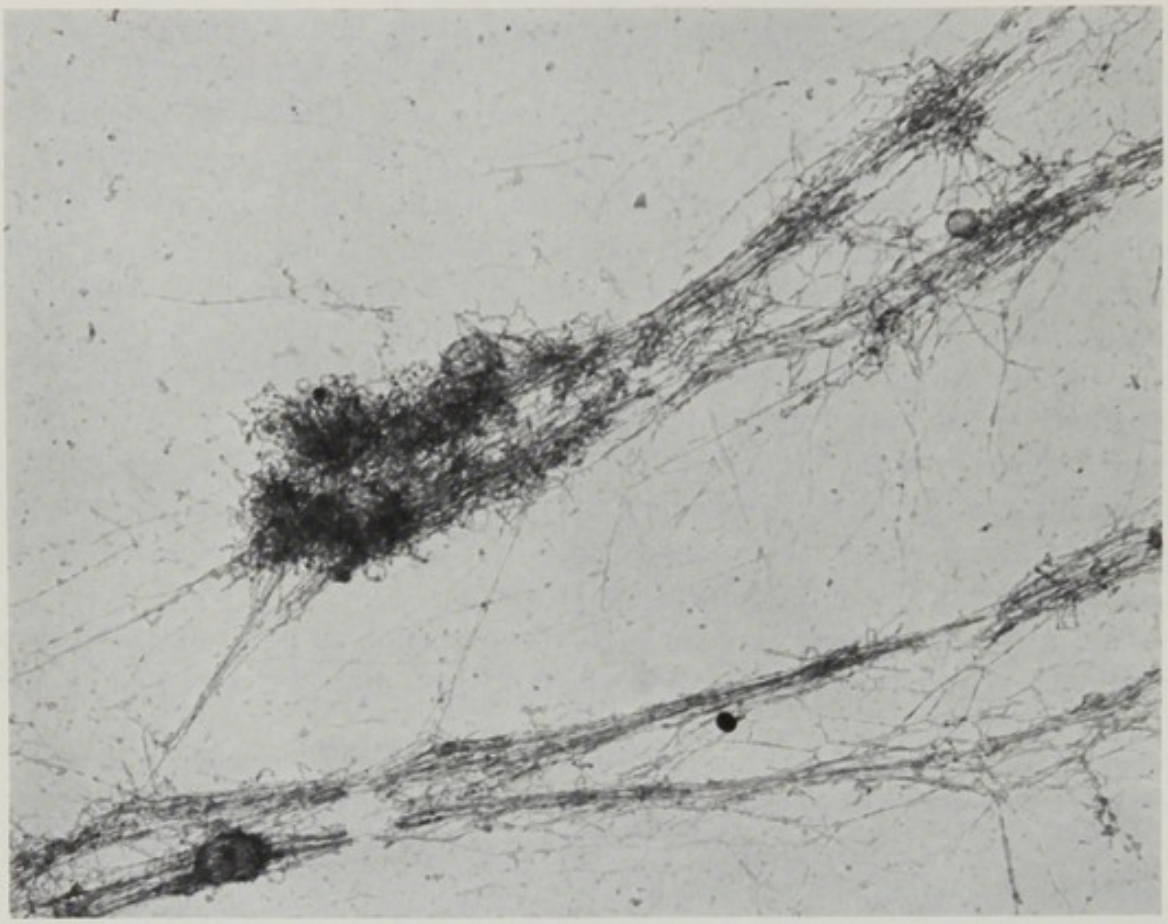


Fig. 7. In this illustration, stretching of a chromosome is shown to reduce the chromomeres. Many of the looping fibers become longitudinal ones. However, not all chromomeres are obliterated in this fashion, as the remaining chromomeric loops show (upper right corner). The chromosome in the lower part of this figure has been overstretched and broken in several places.

affirmative. If a chromosome is extended in the direction of its long axis, it gradually loses its "puffiness." Chromomeres become smaller and thinner (Fig. 7). If further extension occurs, one can observe some broken longitudinal fibers, and the bundle of longitudinal fibers gets ever thinner. Eventually the fibers lose their typical character and are stretched into thinner fibers, which only rarely can be discerned as individuals. When a chromosome contracts-condenses, puffiness increases to the point that nothing in the interior can be discerned. In Fig. 8 the diameter of a round chromatid (Fig. 6) can be seen to have doubled. Few loops are seen leaving the chromatids in any direction, and we are left with the electron microscopic equivalent of a highly condensed chromosome as seen by light microscopy (Bahr, 1970; Fig. 5). This type of chromosome is only rarely found in preparations for electron microscopy, because the method of preparation favors selection of chromosomes with extended fiber loops that serve to anchor the heavier-than-water chromosomes to the denatured protein film at the air–water interface (Fig. 9).

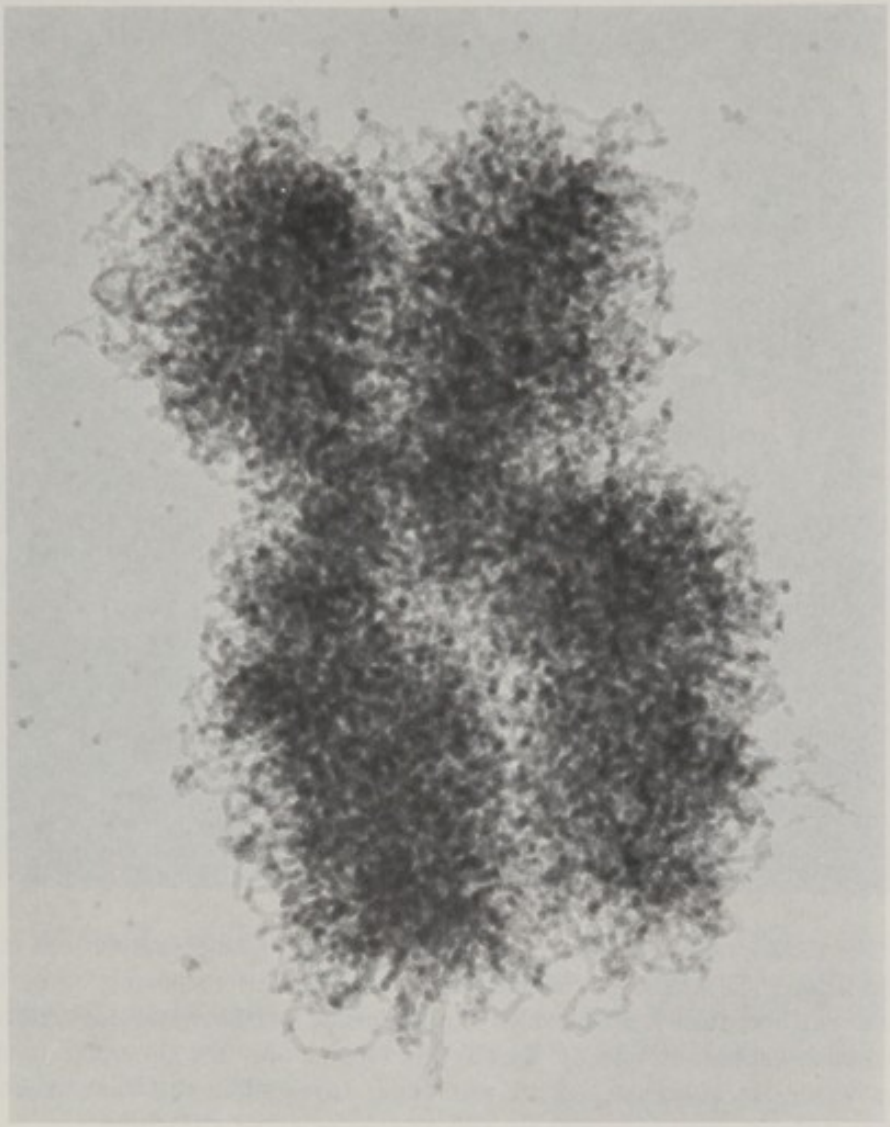


Fig. 8. A fully condensed chromosome is shown. In its periphery only fiber loops can be seen. When compared to Fig 6, it can be seen that the diameter of the chromatid has nearly doubled. This type of chromosome is rarely found in water-spread preparations.

Stretching in the lateral direction (Fig. 10), although not seen as frequently as longitudinal stretching, reveals multiple loops extending from one chromatid to another. This is well illustrated in the early paper of Abuelo and Moore (1969), and indicates the reason subchromatids were once believed to exist. In the middle and telomeric chromomere of the lower chromatid shown in Fig. 10, a dual structure can be seen that was fortuitously produced by the forces of tension. There is no such division in the sister chromatid.

From the principle of symmetry and the fact of mononemy, the reasonable notion follows, that all connections between sister chromatids, including the centromere, are loops, mutually extended from each chromatid and anchored in

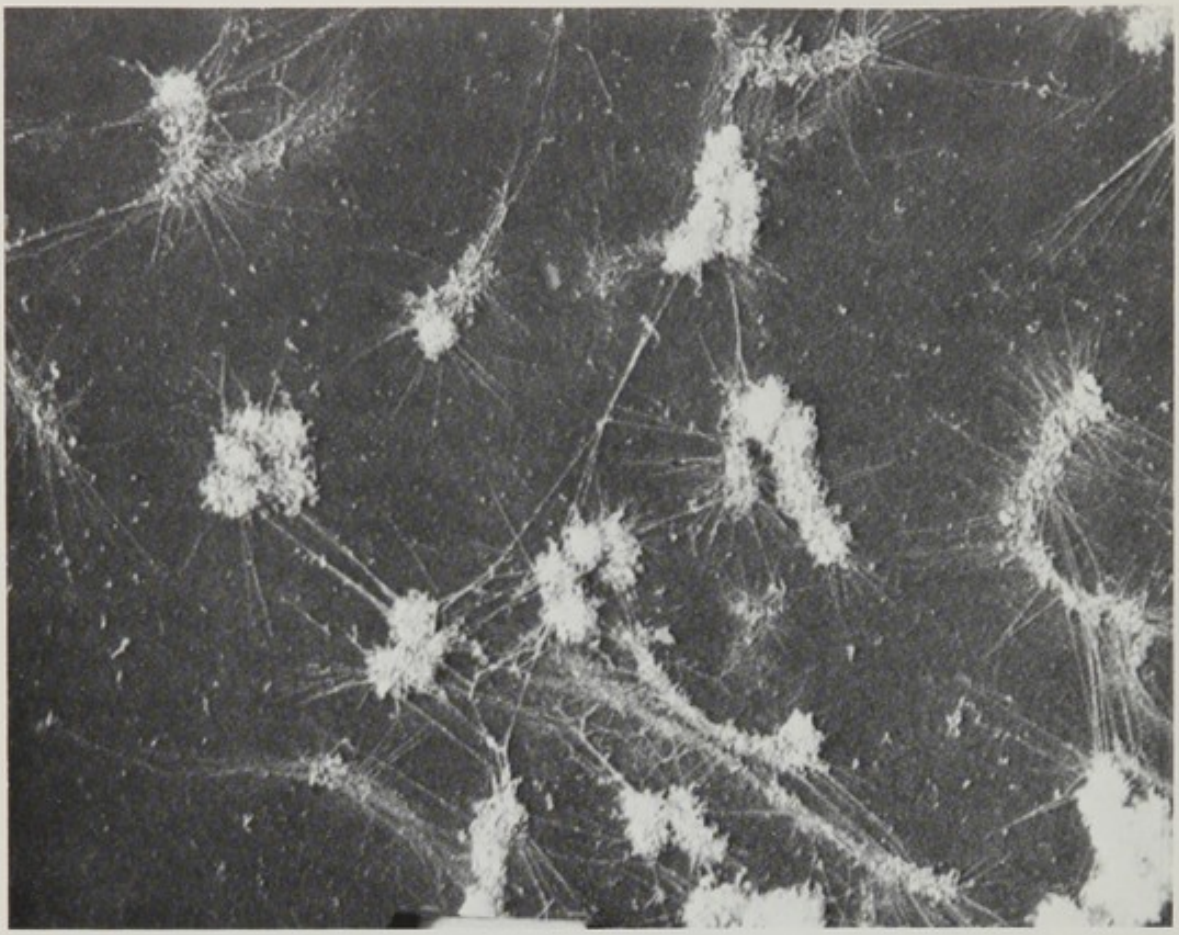


Fig. 9. The reason chromosomes of the type shown in Fig. 8 are rarely seen is presented in this illustration. The scanning electron microscope reveals the many "moorings" of a chromosome which is firmly attached to the underside of the water–air interface of the spreading trough. These moorings are usually the loose loops. They merge into the protein film at the water–air interface and are put under stress when this film expands. Those chromosomes with the most moorings are also most affected by stretching.

the opposite sister chromatid by salt linkages, disulfide bonds, or other protein-protein bonds that are dissolvable by changes in the chromosomal environment (Fig. 11).

A chromosome so constructed, according to calculations from scanning electron microscopy (Golomb and Bahr, 1971), fills about 55% of the volume calculated from an approximation of its surface. Consequently, a chromosome is a delicate and most easily deranged structure, especially in the unfixed state. When, during spreading of chromosomes on water, the surface forces tear at those fibers submersed in the protein film at the interface of water and air, considerable distortion may occur (Figs. 9, 10). Those using shearing forces in the isolation of chromosomes will incur the same derangements, but to a lessor extent, when chromosomes have been fixed. There is a positive side to these

Fig. 10. In this transmission electron micrograph, the stretching forces have separated the two chromatids and have opened up one of them. According to quantitative measurements, both chromatids have the same mass. The lower one shows that there is no core or axial element in mitotic chromosomes. It can be seen why subchromatids have sometimes been described by light microscopy.

destructive forces. In some instances their effect is mild enough to "open up" the interior of a chromosome for inspection. In such opened chromosomes we have found no evidence for the presence of a core or backbone structure. The chromatid is composed exclusively of fibers and their specializations.

It behooves us here to inject that the manner in which whole-mounted chromosomes are prepared (see Section VII) removes the kinetochore. This is presumably because it consists of a lipid-containing membranous material that is extracted in the dehydration series. In thin sections of metaphase cells fixed in osmium tetroxide, the kinetochore can be seen as an osmiophilic laminar structure. This has been very clearly and convincingly demonstrated by Brinkley and Stubblefield (1970; see also Moses *et al.*, 1974).

The fibrous nature of a chromatid is an invitation to reproduce it on a larger scale with man-made materials. This was done, and the result is shown in Fig. 12.

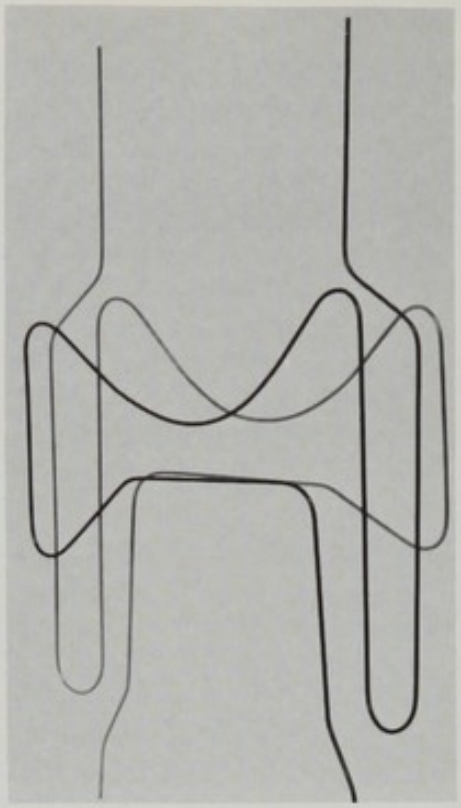


Fig. 11. Model of reciprocal loops somewhere along a mitotic chromosome.

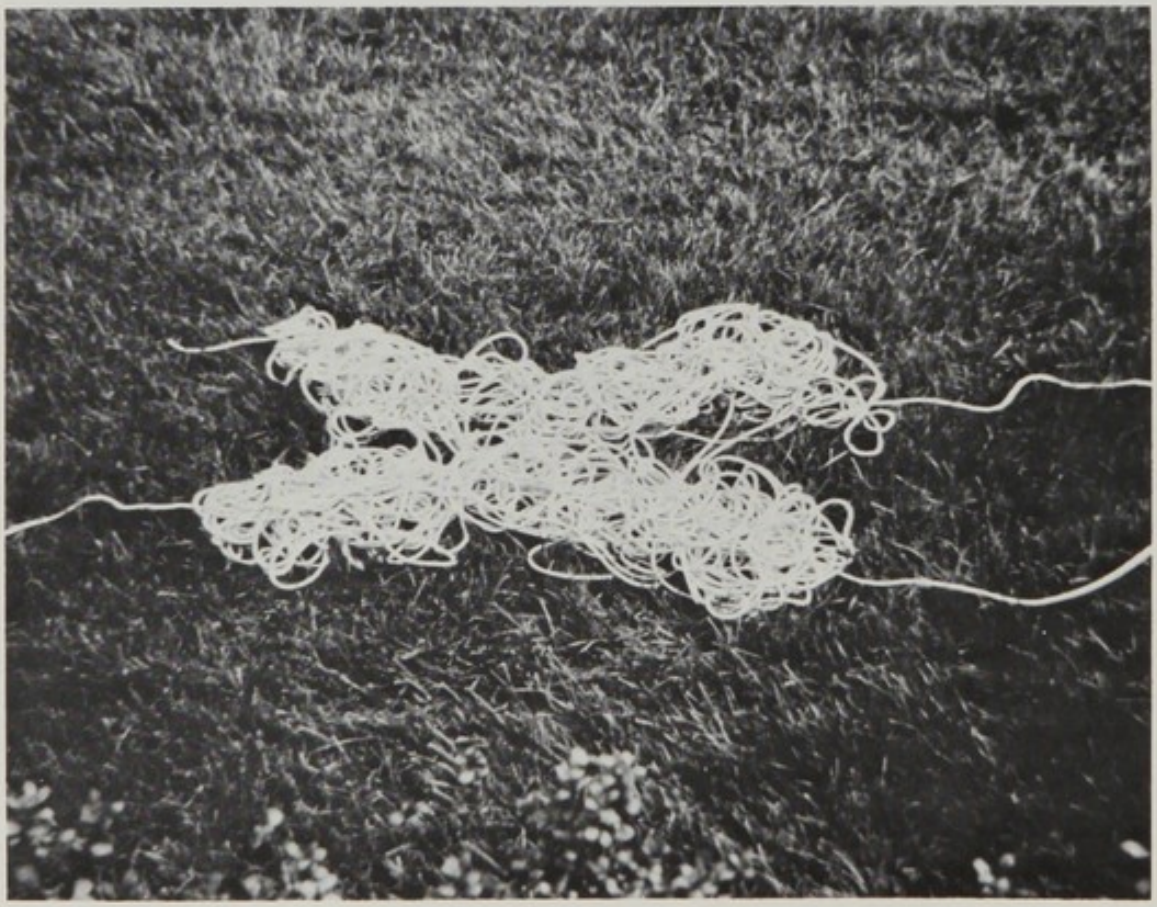
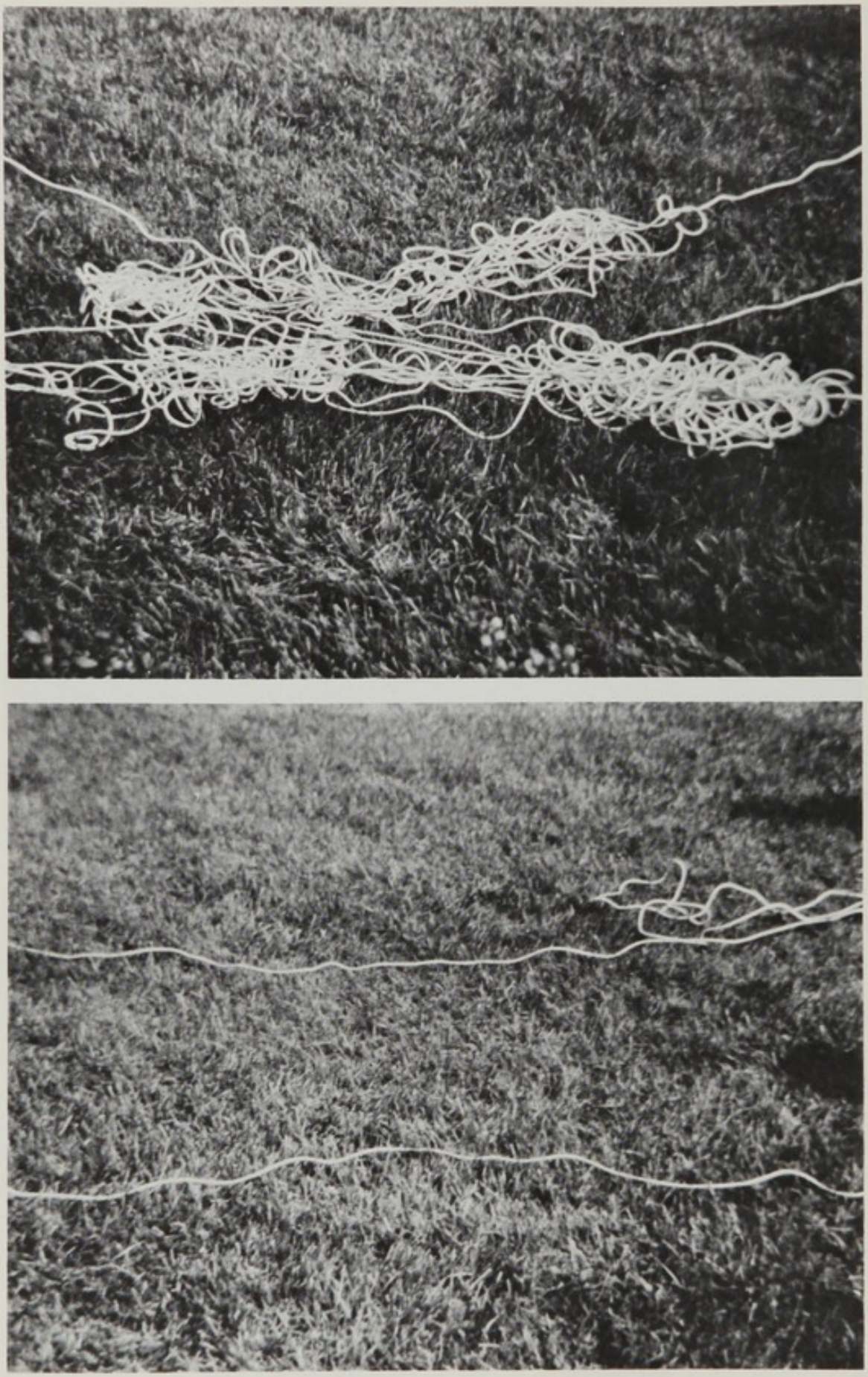


Fig. 12. A "chromosome" has been folded from two clotheslines and is taken out to pasture.

Fig. 13. The properties of the model chromosome become apparent when all protein bonds are broken by enzymes (*ecce*!) or by changes in the ionic milieu.

White clothesline was used to represent chromatin fiber. Some points, where fibers touched were tied with cord to simulate chemical bonds (see Comings, 1968). This introduced some degree of stability in the model. A fiber was left extending from each telomeric end, the significance of which will be discussed further on. The next step in the imagined post anaphase was to dissolve the bonds between fibers (Fig. 13). Now it was easy to demonstrate the nature of a chromosome by taking apart the model. In the subsequent not quite physiological events, the four loose ends were pulled without further touching the chromosome model (Figs. 14, 15). Eventually only two chromatin fibers could be seen. The association of the two sister chromatids, with multiple strands appearing to cross from one chromatid into the other, was thus dissolved without difficulty.

It is easy to realize that this model experiment is merely a demonstration of the probable properties of chromatids, not a sequence of intracellular events. The next illustration (Fig. 16) should be seen in the same vein. It represents the final return of a chromosome to interphase. It is pertinent in a loose sense, because an analogous electron micrograph can be juxtaposed to it (Fig. 17).



Figs. 14 (top) and 15 (bottom). By simply pulling at the ends of each chromatid fiber the whole chromatid can be dissolved and disentangled without ever touching it again. Centromere and interchromatid loops are smoothly resolved.

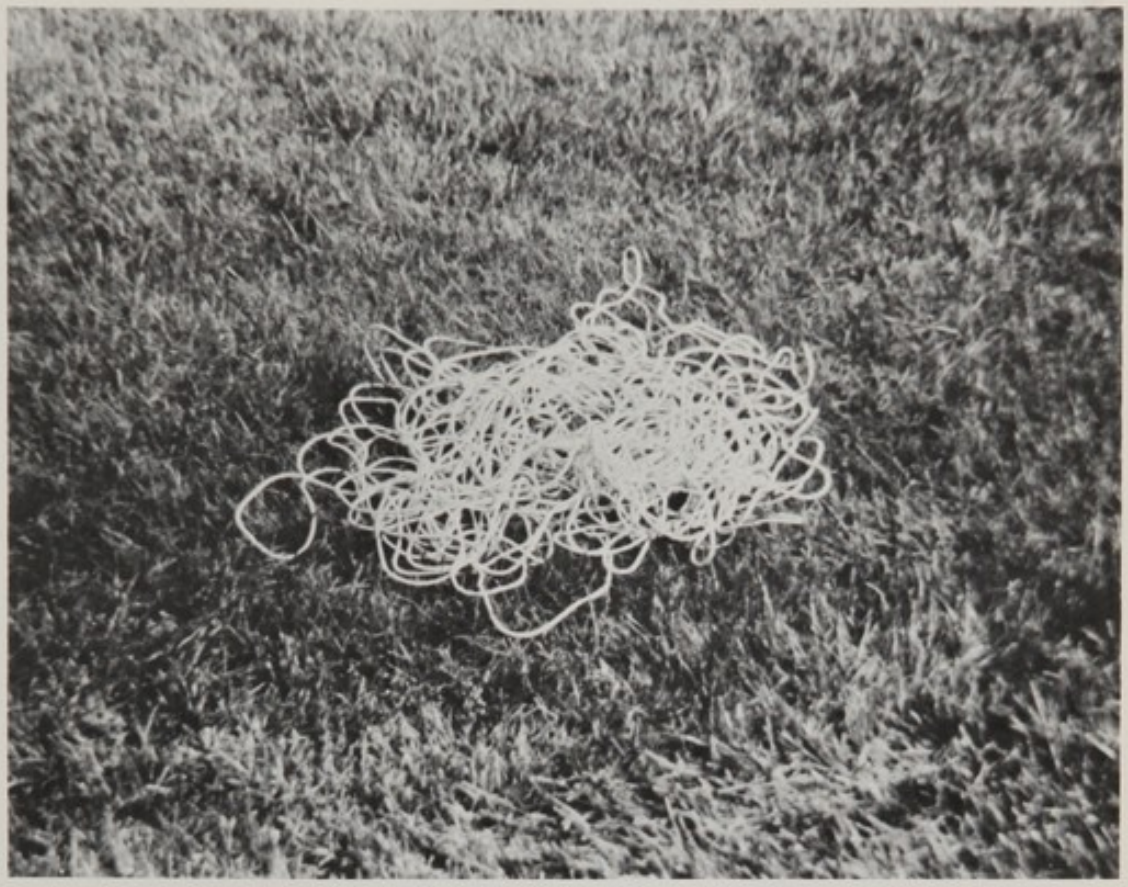


Fig. 16. Eventually one of the clotheslines is gathered to resemble an interphase nucleus. Compare this with Fig. 17.

B. Interchromatid Loops

How can one conceive of the origin of lateral loops from a chromatid and the multiple interchromatid connections? It is probable that the newly synthesized DNA duplexes and chromatin fibers remain physically close until condensation of chromosomes begins at prophase. Prophase must involve complete physical separation of the two fibers over a distance of 3 to 4 μm, i.e., the distance between sister chromatids. Before separation occurs, loops from one chromatid are attached to fibers from the other, as schematically suggested by Fig. 18. A black chromatid pulls at a gray, and a gray chromatid pulls at a black loop. The attachment points are protein bonds, to be dissolved at anaphase. A difficulty arises with this concept. How can a gray loop distinguish between a black and a gray fiber? The only difference conceivable, while maintaining the tenet of absolute comparability of chromatin fibers, may be found in the rate at which proteins are associating with the newly synthesized complementary DNA strands. If this process lags significantly behind the replication fork, then the

Fig. 17. A lymphocyte nucleus at G_1 with innumerable 200 Å fibers emerging at the periphery.

proteins remaining associated with each separated chain of parental DNA molecule would be different because the nucleotide sequence in each is different. This may be the only difference currently imaginable that might provide the means for cross-recognition of sister chromatid fibers.

There are, in fact, pronounced differences in the thymidine content of the two parental DNA chains constituting the double helix. Such sequences are near or in the centromere. Lin and Davidson (1974) were able to show this difference

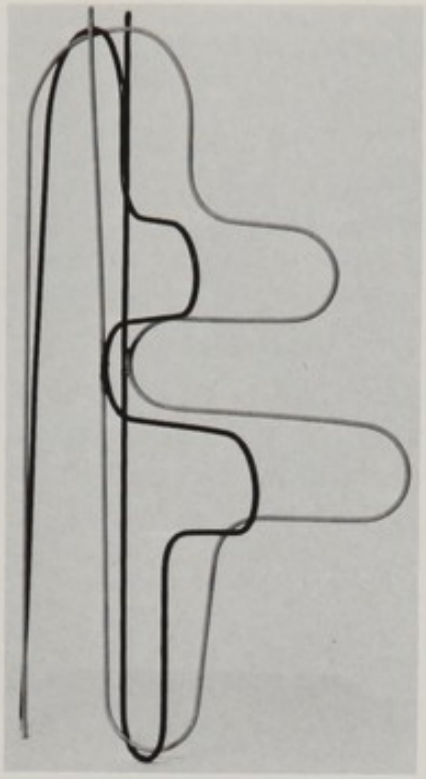


Fig. 18. Wire model suggesting the attachment of a loop from one chromatid (gray) to a fiber from its sister chromatid (black)—the presumed prerequisite for the formation of interchromatidic loops. For explanation see text.

through the use of BUdR (Latt, 1973). They conclude, from the fact that strongly fluorescing spots were located on opposite arms of metacentric chromosomes after one replication cycle, that these are indicative of a definitive polarity of DNA at the site of the (evolutionary) centric fusion and of continuity of DNA through the centromere.

Cross-recognition and establishment of a centromere loop may also serve to turn the originally parallel strands by 90 degrees to face each other and bring the chromatids into the symmetry described.

Interchromatidic loops not only explain spurious *isochromatid labeling* in experiments with tritiated thymidine but also the manner in which *fragments and minutes* stay together without the benefit of a centromere.

C. Interconnections of Chromosomes

When first seen in the electron microscope, some chromosomes were connected by fibers of an appearance and mass per unit length comparable to the fibers in the chromosomes themselves (Lampert *et al.*, 1969). More recent electron microscopic studies in this laboratory have shown these fibers to be from 4 to 20 μm long. Since the length often exceeds the diameter of a human

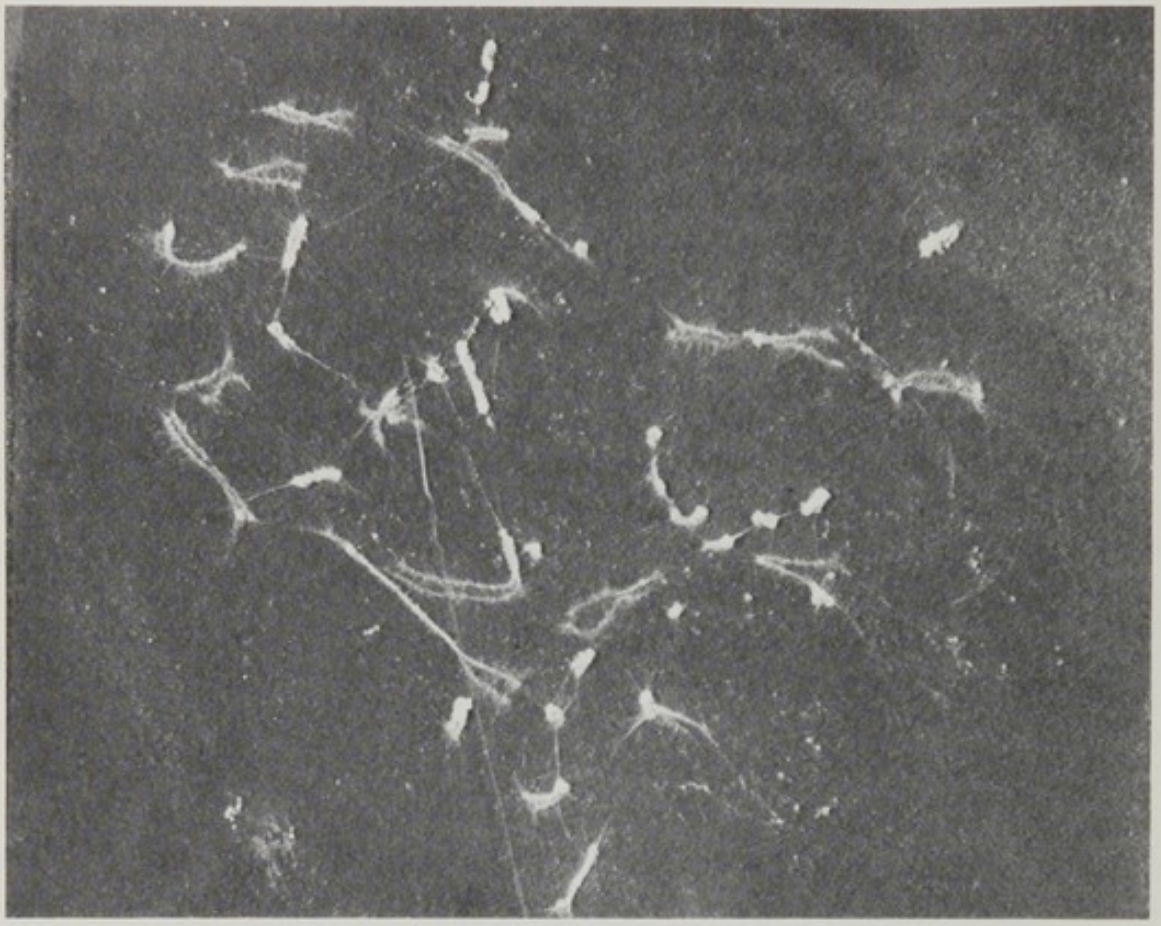


Fig. 19. This scanning electron micrograph demonstrates some of the long, interchromosomal connective fibers that are plainly seen when coated with a conducting layer of gold.

lymphocyte nucleus, it must be assumed that the interconnections are normally folded. Interconnecting fibers have also been observed in the scanning electron microscope (Golomb and Bahr, 1971, 1974b) and are apparent in Fig. 19.

A chromosome condenses gradually to the compact metaphase state. Long loops are folded and retracted into the body of the prophasic chromatid, predominantly at the locations of the chromomeres (Bahr, 1973a). The loops are found in the immediate vicinity of the chromatid and very often are entangled with other chromosomes. Figure 20 renders a vivid picture of such a prophasic enmeshment and the difficulties involved in smoothly disentangling loops at metaphase. Such *interchromosomal fibrous associations* persist in many preparations for electron microscopy (Fig. 21) and are a common feature of metaphases in light microscopy (Takayama, 1975). They may be the reason some aberrations, such as dicentrics, could falsely be interpreted as translocations. In the frequently observed "stickiness" of chromosomes (Beadle, 1933) there is difficulty to resolve enmeshment, presumably because of chemical effects on chromosomal proteins. Pathak and McGill (1974) have recently described these

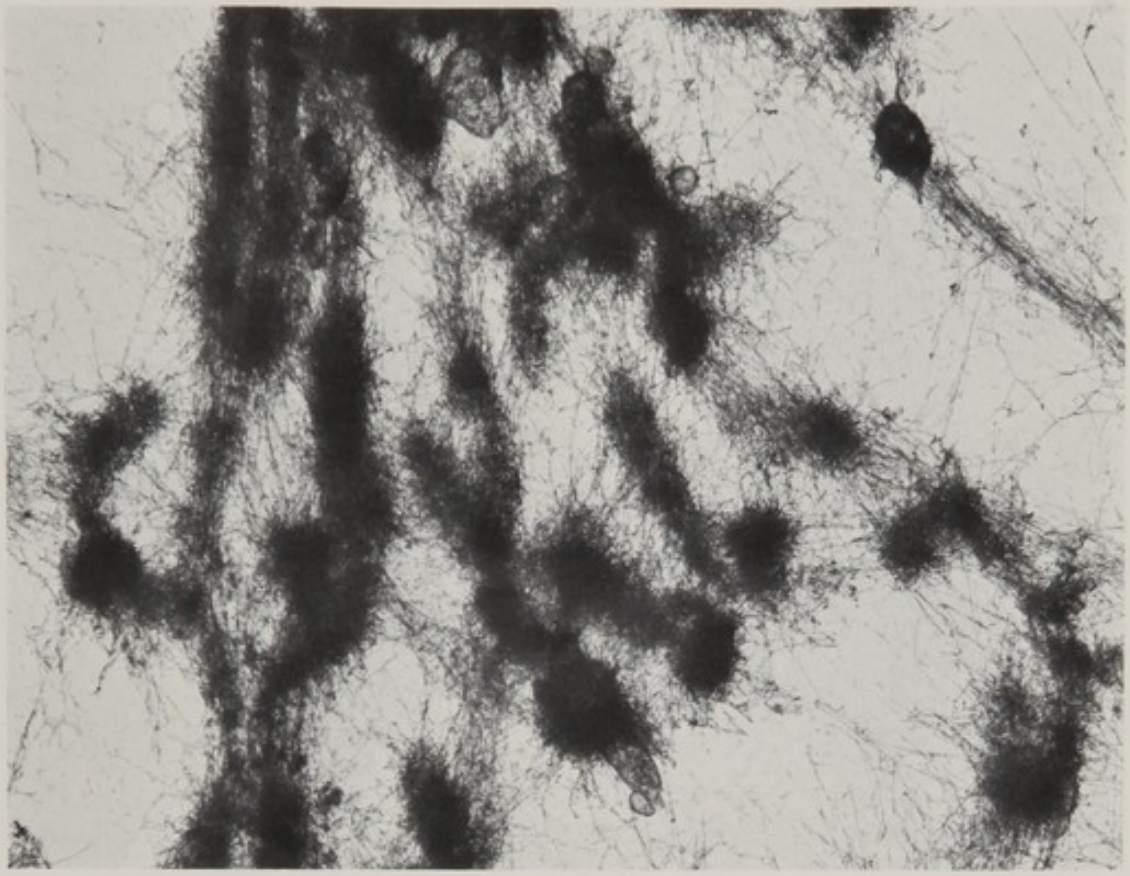


Fig. 20. Interchromosomal fibrous associations in a late prophasic nucleus. Extensive enmeshment of fibers between chromosomes and between chromatids can be seen.

fibrous connections and the difficulties in separation of sister chromatids at anaphase.

Interchromosomal fibrous associations should not be confused with *chromosomal connective fibers*. The latter are fibers that clearly link two chromosomes over distances several times the size of a chromosome. Although they are most often seen to emerge from telomeres, fibers have been found to connect to all other parts of a chromosome.

Let us return for a moment to the model of a folded fiber. It is clear that mononemy and genetic polarity predicate the concept of a beginning and an end to a chromatidic fiber, regardless of whether each chromatid is a closed molecular DNA circle or whether the beginning and the end are located on connective fibers. Because long interconnective fibers appear in the electron microscope to enter and leave a chromosome, the fibers were called afferent and efferent, implying polarity (Bahr, 1975). For the afferent fiber it was proposed to write "→ 21" if, for example, one talks about chromosome 21. The efferent is analogously written "21 →." In the case of a translocation (Fig. 22) one would write "→ 15/21 →." From each chromosome four fibers emerge in pairs, two

Fig. 21. Some remaining fibrous associations between two fully condensed chromosomes. These fibrous associations are often extensive enough to be seen under the light microscope. Because Beer's law governs the absorption of light by dyestuffs, the body of a chromatid appears to contain a disproportionately greater amount of stain than the fibrous associations. Similar considerations apply to band and interband areas.

afferent and two efferent. In most instances the pair emerges as separate fibers, shortly thereafter to appear twisted around each other. Quantitative measurements reveal that the long and more visible interconnective fibers possess double the mass of a single fiber.

DuPraw (1970) reviewed diverse observations from the literature in the light of chromosomal interconnectedness, especially radial metaphase configurations and the possible influence of interconnectives on translocations and the resulting speciation. Since then, much additional, though indirect, evidence for linkage of chromosomes by "forces," i.e., fibrous connections, has accumulated. For example, the sex chromosomes in *Microtus agrestis* are found in constant apposition or opposition at metaphase and telophase and are stable throughout the cell cycle (Pera and Schwarzacher, 1970). Costello (1970) added to the literature on radial metaphase configurations by presenting a micrograph of a circular pattern of alignment, group by group, of corresponding chromosomes. In working with *Muntiacus muntjak*, Heneen and Nichols (1972) observed a similar positional

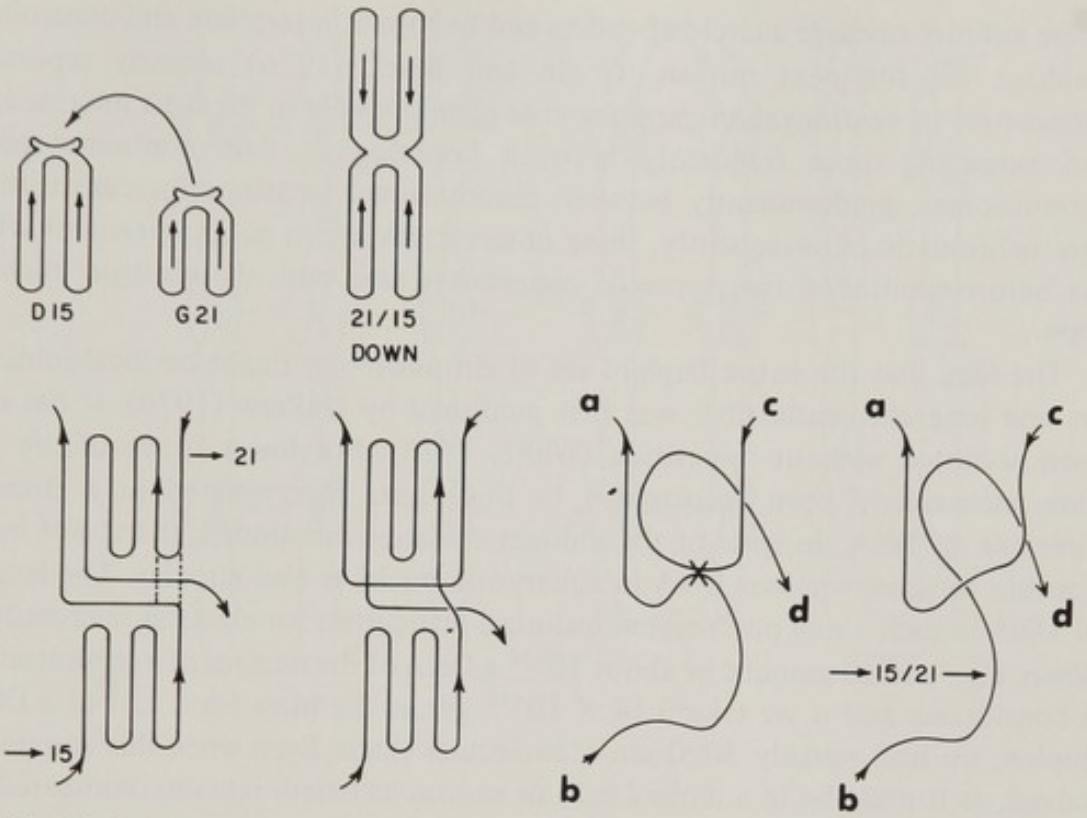


Fig. 22. Schematic representation of a single exchange at interphase producing a 21/15 Robertsonian fusion. Polarity has been arbitrarily assumed for both chromosomes as indicated by arrows. Both chromosome 15 and chromosome 21 have afferent fibers (→ 15, → 21) and efferent fibers (15 →, 21 →). A very much reduced folding pattern for both chromosomes makes it easier to understand where the exchange must take place in order to integrate 21 into 15. Because of chromosomal connective fibers, chromosome 15 is interspersed between afferent fiber b and efferent fiber a, and chromosome 21 between afferent fiber c and efferent fiber d. After the exchange, → c is linked to a →, and the new chromosome 15/21 is situated between → b and d →. Reprinted from G. F. Bahr (1975). *Fed. Proc.* **34**, 2209–2217.

regularity at metaphase. We will discuss this finding at a later point in some detail. Similar observations on associations and regularities were made by Barton *et al.* (1965), Back and Zang (1969a,b), Ferguson-Smith and Handmaker (1963), Galperin (1968, 1969), Grumbach *et al.* (1963), Miller *et al.* (1963a,b), Morishima *et al.* (1962), Ockey (1969), Zang and Back (1967, 1968), Rosenkranz and Fleck (1969), Evans (1974), Emmerich (1973), Hoo and Cramer (1971), and Heneen and Nichols (1972). Long-range connections between chromosomes are also visible in Fig. 1 of the fundamental paper by Hoskins (1965), who isolated chromosomes by microsurgery. In this context it is of interest that Kraemer *et al.* (1972) invoke the concept of DNA continuity, at least between some chromosomes, in a general explanation of what may be called the "Los Alamos paradox," namely, the striking finding that total DNA content remains constant although the number of chromosomes varies in heteroploid cell lines.

These authors envisage a circle of end-to-end linkage at interphase and controlled breakage for the next mitosis. Godin and Stack (1976) recently reported connectives in *ornithogalum* chromosomes plainly visible in the light microscope and extending more frequently between homologous, than nonhomologous chromosomes, predominantly between chromosomal locations for constitutive heterochromatin. Consequently, these observations are in good agreement with the before-mentioned two types of connectives seen with the electron microscope.

The idea that the entire haploid set of chromosomes might be local foldings on one long chromatin fiber was first published by DuPraw (1970). It has not been accepted without opposition (White, 1973). For lower forms of life we have increasingly been accustomed to find genes incorporated in a circular molecule of DNA. In spite of the indirect evidence mentioned, it has not been possible to show whether DNA in eukaryotic nuclei is also circular. The length of DNA in such a ring poses severe technical difficulties for electron microscopy. When, e.g., a small amount of about 10^{-14} g DNA of the nucleus of a chromosome is considered, and if we take 3.14×10^{-18} gm as the mass for 1 μm of a DNA duplex, we find roughly 3000 μm of molecular chain. Even when this amount is halved, as it must be in a diploid cell, an enormous length remains, compared to the dimensions of an opening in an electron microscope specimen support. A grid opening with the unusual side length of 500 μm is large with respect to the tensile strength of any support material (carbon, silicon monoxide, gräphite, and the polymers). Any increase in thickness, i.e., tensile strength, decreases the relative contrast of the object. An entirely new methodology will have to be introduced to visualize whole eukaryotic DNA molecules or to identify unique nucleotide sequences in pieces of the molecule for later reconstruction of the whole. Until that time we may extrapolate from our knowledge of lower forms and assume that eukaryotic DNA also occurs in ring configurations. Even though the concept of interconnectedness of chromosomes has strong indirect support from various observations, it remains an unproved but logical conclusion that the fiber within a chromatid is continuous with afferent and efferent fibers. Haapala and Soyer (1974) found *chromatids* of the polytenic chromosome of two species of dinoflagellates to contain circular DNA.

In view of the very suggestive electron micrographs and numerous light microscope observations, the concept of large circles of DNA in which chromosomes represent localized foldings deserves serious consideration. In mitosis, difficulties can arise when the attachment of spindle fibers to kinetochores has equal probability for each side, i.e., when spindle fibers attach regardless of rotation of the chromosome around its long axis. In Fig. 23 an attempt is made to illustrate chromatid separation under various conditions at mitosis. In A a piece of the presumed circle with three chromosomes is drawn. No difficulty is encountered in the separation of chromatids (arrows indicate direction of

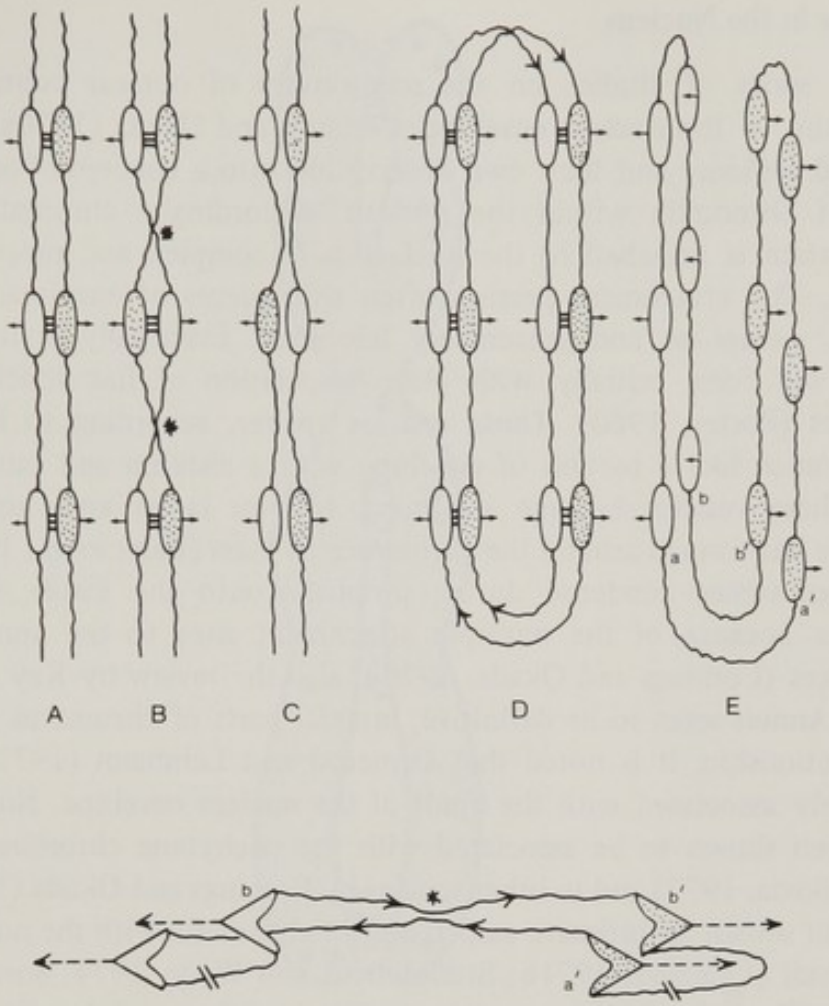


Fig. 23. This drawing is intended to explain some of the consequences and difficulties associated with chromosomal connective fibers. For explanation see text.

spindle fibers). Sister chromatids are left white or hatched for clarity only. In B the middle chromosome has rotated. Because of inherent polarity, only one type of fiber exchange can take place. The site of an exchange is marked with an asterisk. At C the separation of "mixed" chromatid chains is suggested.

When a telomeric reciprocal exchange has taken place (Bahr, 1975; model of sister chromatid exchange), there will remain an unresolved topologic problem of separating chromatid chains D and E. At mitosis, chromatids a and b are still connected to a′ + b′, as illustrated in the lower part of Fig. 23. The fibers connecting a with a′ and b with b′ possess opposite translational polarity; that is to say that any exchange will close the parting circles a to b and a′ to b′. It is conceivable that the vigorous movements of chromosomes recorded by Bajer and Molè-Bajer (1963) bring unresolved connective fibers in apposition for the resolving exchange, whereby exchange sequences of the same or similar type facilitate exchange, as they would in sister chromatid exchanges.

D. Order in the Nucleus

In a series of studies on the relationship of nuclear content, especially chromatin, to the nuclear envelope, Comings and Okada (1970a,b,c) solidified scattered evidence and their own observation into a concept of orderly arrangement of chromatin within the nucleus. Accordingly, chromatin in the dispersed state is attached to the nuclear pore complex and most likely to the annulus. The attachment persists often with pieces of envelope still attached through metaphase and presumably telophase. Eventually a new nuclear envelope will form initially with close association of flat vesicles with chromosomes (Porter, 1960). These vesicles appear, according to Porter's micrograph, much like a section of envelope with a cisterna and outer membrane. Later these vesicles become confluent to ever larger areas until the entire envelope has formed around the chromosomal mass (Barer *et al.*, 1960).

Chromosomes condense during prophase onto the inside of the nuclear envelope because of the multiple attachment sites to the annuli of porous complexes (Comings and Okada, 1970b; also the review by Kay and Johnston, 1973). Annuli seem to be definitive, integral parts of chromatin. In evidence of this relationship, it is noted that Ormerod and Lehmann (1971) found DNA intimately associated with the lipids of the nuclear envelope. Numerous annuli have been shown to be associated with the pachytene chromosomes of *Omocestus* (Sorsa, 1972) and in other species by Comings and Okada (1970b). Others have also shown an intimate association of chromatin with the nuclear envelope and annuli (Lampert, 1971b; Stubblefield and Wray, 1974; Sorsa, 1973). The most striking observations in this respect are those of Aaronson and Blobel (1974) who show that the pore complex does not require the membranes of the envelope for attachment to nuclear chromatin or for structural integrity. Annuli remain on chromatin (fibers) while membranous parts of the envelope are dissolved by detergents. The old observation by histologists and pathologists of chromatin condensing onto the nuclear envelope when fixed with formalin is explained by this relationship. Electron microscopists have seen how aldehydic fixatives produce margination of chromatin, while osmium tetroxide does not. Margination of chromatin is also observed in many pathologies.

Our own observations confirm those of previous students of envelope-nuclear pore-chromatin relationships and further indicate that the attachment sites along a chromosome are specific in their location. The positions of these sites suggest that chromosomes may have evolved from smaller functional units comparable in size to minutes, each with a distinctive attachment pattern. We base this suggestion on the observation of a symmetrical appearance of attachment sites for annuli along sister chromatids. A composite of observations is embodied in Fig. 24. One or two annuli have been found incorporated into loops at the site of a chromomere. Maul *et al.* (1972) in a freeze-etch study of HeLa cells and lymphocytes counted about 2000 annuli in the envelope of unstimulated nuclei

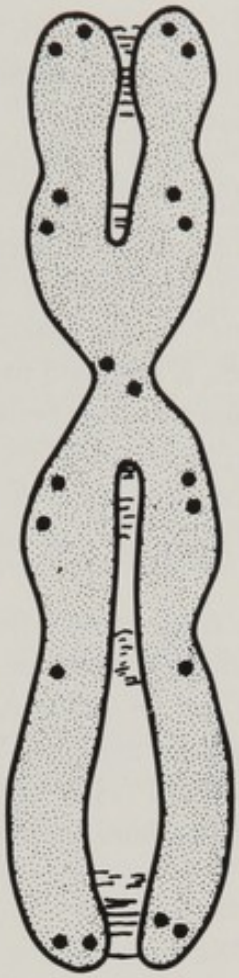


Fig. 24. A summary of some observations of the position of nuclear pores (annuli) on a chromosome. This is an artist's rendition of a light micrograph of a submetacentric chromosome with typical modulation of shape in long and short arms. At crucial points, in particular at the telomere and close to the centromere, interchromatid loops make for weakly stained bridges between chromatids.

and about 4000 annuli in prophasic nuclei. At 500 chromomeric bands per haploid set of chromosomes, there are 1000 chromomeres in a diploid human complement. Dividing Maul and collaborators' figure of 2000 by the number of chromomeres in a G_1 nucleus, we calculate two annuli per chromomere. This is in good agreement with our observation.

Maul and colleagues show that DNA synthesis lags behind the increase in the number of annuli per nucleus, but catches up, reaching the G_1 ratio two-thirds of the way through the 48 hour cycle. Protein synthesis keeps step with the increase in annuli throughout the full cycle. We deduce from these observations that the means for folding or coiling DNA and its orderly attachment are provided prior to replication of the bulk of DNA.

We surmise that the attachment of every chromomere has evolutionary significance, and that it assists in distributing chromatin in an orderly manner

after telophase. The membrane, forming with annuli as points of initiation, provides the basic orientation and possibly some force in the process of transition from telophase to interphase. The nuclear envelope may also provide the chromosomal element with a special functional unit, the kinetochore. In protista, the convergence of spindle fibers onto a thickened part of the nuclear envelope has frequently been observed. Is this special property of envelope perpetuated in those portions of the nuclear envelope that develop in close association with the chromosome? Is the kinetochore, in reality, a part of the nuclear membrane complex which is attached to the centromere by centromeric fibers which are attached to the nuclear membrane during interphase?

Recent studies by W. Franke, Heidelberg (personal communication), suggest however that the attachment of chromatin to the inner nuclear envelope is tenuous at best and in certain stages of the cell cycle the nuclear envelope is entirely free of chromatin.

Order in the nucleus emerges from various findings. It is maintained through association and merger with the nuclear envelope. Inside the nuclear space, order prevails that in some instances can be observed by light and electron microscopists. From his own observations and review of the literature, Lafontaine (1974) presented evidence consistent with the notion that in many plants, chromosomes persist in an orderly manner as filamentous, skeinlike structures in reticulate interphase nuclei.

V. RELATION OF CHROMOSOME STRUCTURE TO THE BANDING PATTERN OF CHROMOSOMES

We have seen in the preceding sections how the long fiber of a chromatid is folded into longitudinal and chromomeric elements. Under the light microscope acid-fixed and Giemsa-stained chromosomes appear however as relatively solid rod-like bodies. This concept of a chromosome therefore is reflected in many schematic drawings of the literature. Even electron microscopy of thin-sectioned chromosomes did not change this view. It remained for the banding techniques of light microscopy to demonstrate the intricate pattern into which the length of a chromatid is subdivided by chemical affinities for dyestuffs (see the reviews by Schnedl, 1974, and Comings, 1975). At the same time it became apparent that prophasic chromosomes in whole-mounted preparations for electron microscopy showed characteristic discontinuities of chromatids in that the fibrous chromatin was accumulated in "chromomeres," analogous to the beadlike chromomeres of pachytene bivalents in meiotic prophase. A discontinuous symmetrical distribution of chromatin along chromatids can also be seen in the micrographs of Watanabe and Tanaka (1972), although these authors fail to see a connection with chromosome bands.

The chromomeres in human chromosomes and in *Muntjak muntjacus* were studied by densitometric tracing of electron micrographs of unstained preparations (Bahr et al., 1973; Bahr and Larsen, 1974; Green and Bahr, 1975). Each chromosome in a complement possesses an identifying sequence of large and small chromomeres. A total of about 500 chromomeres can be distinguished in a haploid set of human chromosomes (Bahr *et al.*, 1973). An equal number of Giemsa bands have recently been resolved by light microscopy (Yunis and Sanchez, 1975). In accordance with the most recent nomenclature for bands (Paris Conference, 1972), both positively staining bands and interbands are given numerical identity. This may be helpful in describing translocations and other alterations of chromosomal structure, but the underlying assumption of the equivalency of positive and negative bands cannot be supported by electron microscopy, because positive bands are chromomeres—frequently several in one band—while interband or interchromomeric areas are predominantly composed of longitudinal fibers.

It is of interest to ask if chromomeres of human chromosomes represent sites of single genes, as is the case for the natural bands in chromosomes of dipteran salivary glands (see the extensive review by Beermann, 1972). For the human genome, no hard data on the total number of structural genes is available, but some well-reasoned guesses estimate this number to fall between 10^4 and 10^6. Let us for the sake of this consideration assume that 3×10^5 genes exist (Ohta and Kimura, 1971), and, when incorporated into 500 chromomeres, 600 structural genes would be located in an average chromomere. Since in a late prophasic chromosome about 50 fibers are found by a transecting densitometric scan of an average chromomere, and 8 fibers are longitudinal, there are 30–40 fiber profiles left to accommodate 600 genes, or 15–20 genes per chromomeric loop. This discussion is not meant to give the impression that there are grounds to assume where genes are located, but it does provide some figures to underscore the vast difference between dipteran and mammalian chromosomes, in respect to numbers of genes per visible unit. The speculation by Yunis (1974) and by Judd and Young (1973) that structural genes cannot be located in darkly staining Giemsa bands because the bulk of repetitive DNA is located there (Sanchez and Yunis, 1974), should be modified to the effect that structural genes are not excluded when large amounts of repetitive DNA occurs in a chromosome part, since this occurrence may be a normal and necessary adjunct to the performance of structural genes.

We have previously estimated that only about 55% of chromosomal volume is fiber. The chromomeres possess enough flexibility to associate themselves with neighboring chromomeres. Interchromomeric associations and the resulting variation in chromomere positions provide some reasons why structural banding allows for the individual identification of unfixed, single chromosomes under the electron microscope only with some difficulty. This is illustrated by the position

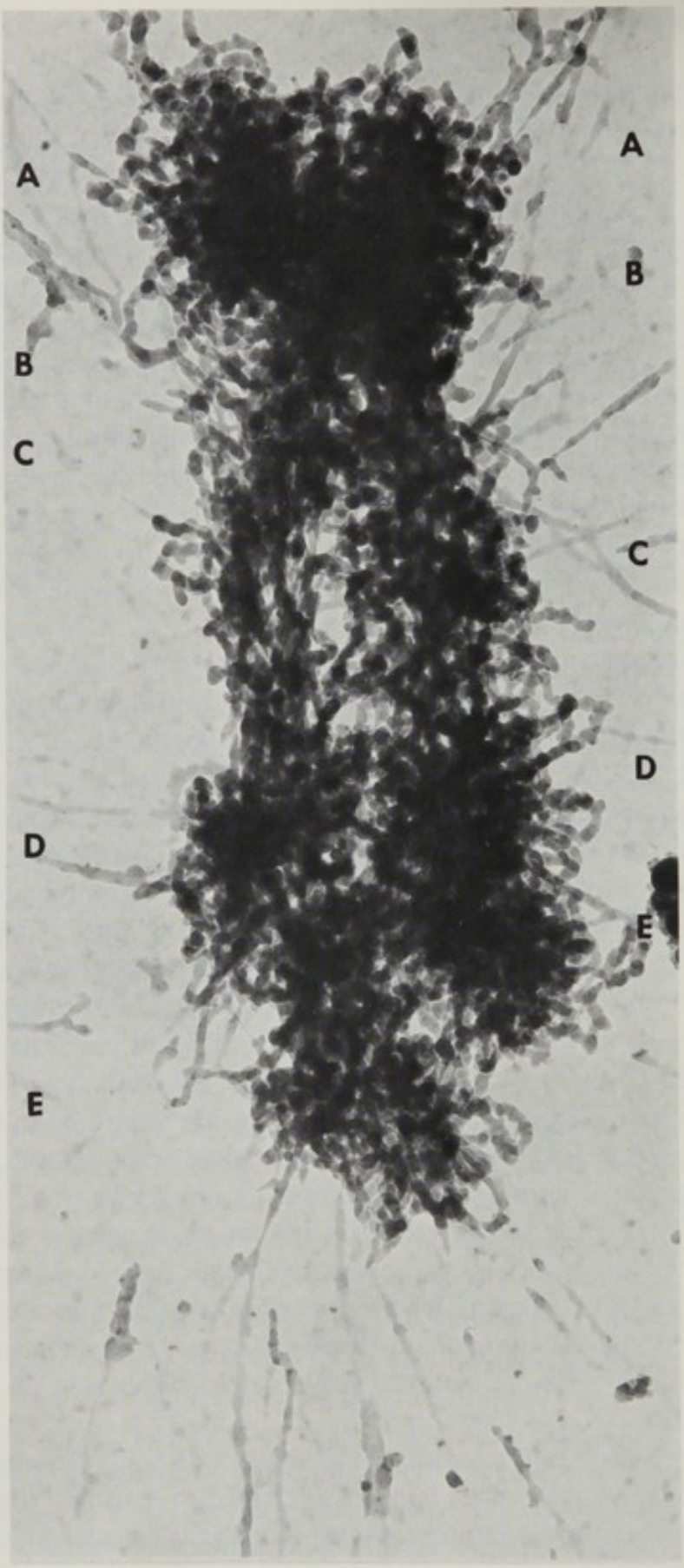


Fig. 25. In this electron micrograph one chromatid is fairly extended, the others less so. Chromomeres are displaced in the extended chromatid because more fiber loops have pulled on it than on the other chromatid. A, B, C, D, and E indicate corresponding loci in the sister chromatids.

of analogous chromomeres in the chromosome of Fig. 25 (Bahr and Larsen, 1974). When chromosomes are fixed *in situ* with acetic acid, not only is histone H1 removed, but the fibrous structure is frozen in its original shape by coagulation of proteins. This is of some help in banding chromosomes for light microscopy, but the variability of band position is still considerable (Selles *et al.*, 1974; Mainguy *et al.*, 1975).

A spiral substructure throughout a whole chromatid has often been described. Such observations are partly due to the oblique arrangement of chromomeres around an imaginary chromatid axis to which many longitudinal fibers roughly conform (Bahr *et al.*, 1973) (Fig. 26). Under the light microscope or in a defocused electron micrograph, a strong impression of a spiral structure is produced (Makino, 1936; Manton, 1950; Ohnuki, 1968), which may change reversibly with treatment (Takayama, 1976).

From the viewpoint of the fibrous structure of chromosomes, there are three reasons for the banding produced for light microscopy:

1. There is a structural basis for banding in that the distribution of chromatin in a chromatid is not continuous, but modulated by the presence of chromomeres.

2. There are specific chemical affinities distributed discontinuously over a chromatid. They may or may not coincide with chromomeres. These affinities may pre-exist in the nucleotide sequence (Weisblum and De Haseth, 1972) or be induced by pretreatment of the chromosome. Brown *et al.* (1975) offered a solution to the question of how Giemsa banding is produced by showing that acid fixation removes H1 and H2 histones, thereby permitting staining. When histones are put back on fixed chromosomes, the staining reaction is completely abolished. H1 has a more decisive role in this respect than H2. Holmquist and Comings (1976), however, provide evidence that this type of blocking Giemsa staining is nonspecific.

3. Upon extraction with alkali or digestion with enzymes, those remnants of a chromosome that resisted the strength or duration of the treatment are stained. Comings and Tack (1973) have discussed this aspect of banding in some detail.

Mitotic chromomeres reappear in a matching pattern at meiosis (Ferguson-Smith and Page, 1973; Comings and Okada, 1975).

VI. ABERRATIONS REFLECTING THE FIBROUS NATURE OF CHROMOSOMES

In this section the most common chromosomal aberrations will be listed with annotation of the mechanism that may reasonably have led to them. Each will be considered from the viewpoint of mononemy of chromatids and continuity

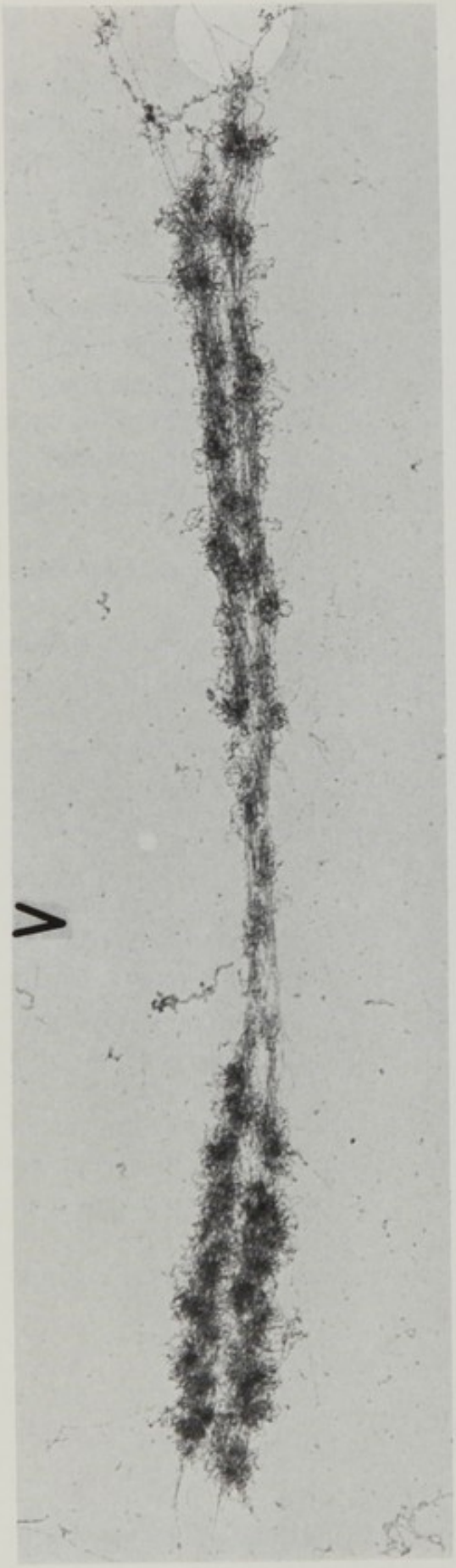


Fig. 26. This micrograph is presented in order to convince the reader that the orientation of chromomeres relative to the imaginary chromatid axis may produce the impression of a spiral. If the viewer squints while inspecting the chromosome, the impression of spirals becomes especially strong at places. The micrograph should, in addition, serve to demonstrate the positional variability of chromomeres. Note that some chromomeres appear to be located off the axis. Arrow indicates location of centromere.

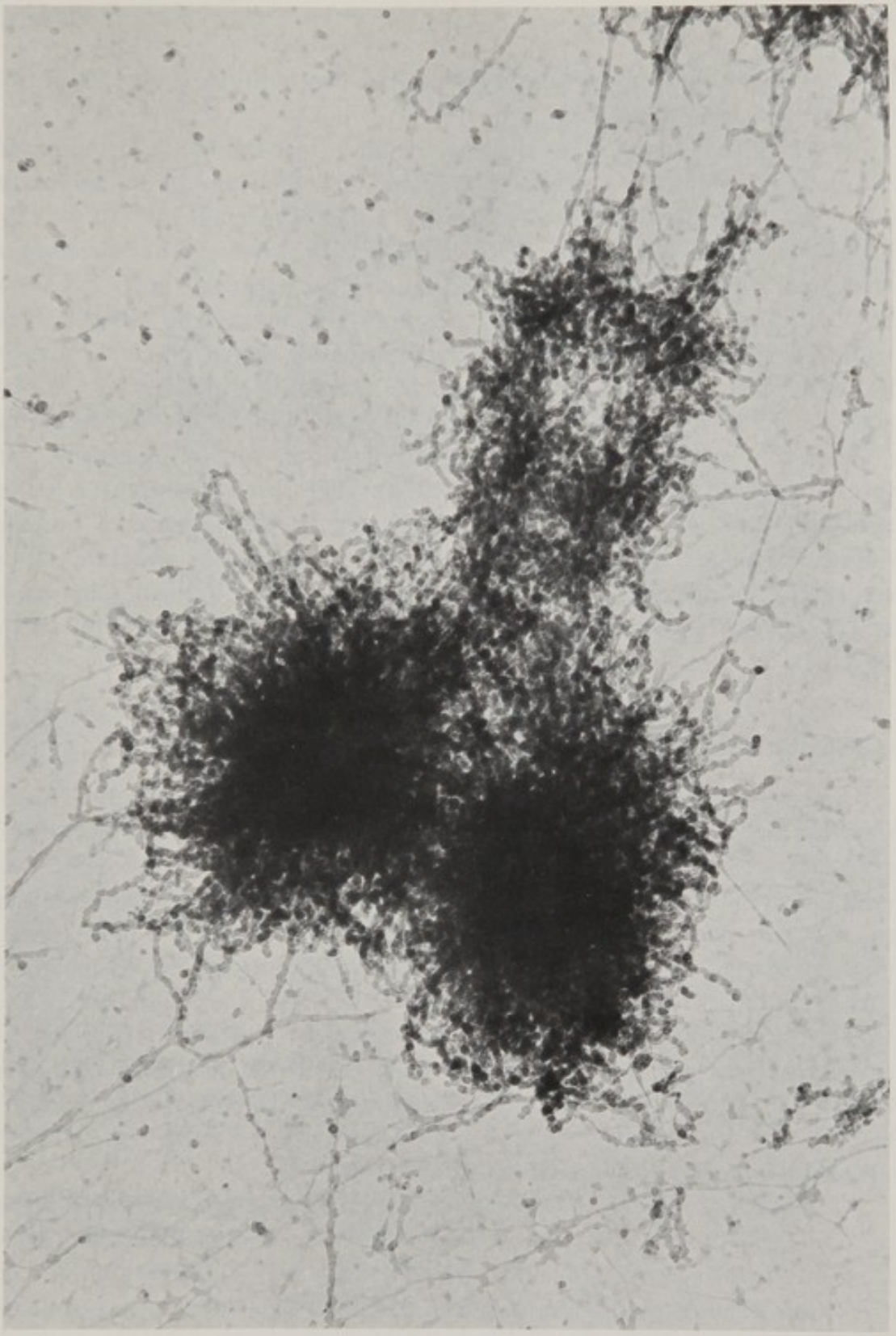


Fig. 27. A small G-group chromosome illustrates once again the complexity of chromosomes. Note the satellites and their extensions, which have the appearance of horns. Translocation or sister chromatid exchange occurs in this mass of fibers with the precision of clockwork and does not disturb the familiar appearance of a chromosome.

of interchromosomal fibrous connections (the continuity of a chromatin fiber over several chromosomes).

According to current concepts, DNA within a fiber can be translated in only one direction. However, a structural gene could theoretically be turned around in the long molecule of DNA of which it is a part. The handedness of the DNA double spiral would not pose difficulties for this event. The direction of translation would depend on the orientation of a particular gene. DNA synthesis is known to proceed in two directions (Huberman and Attardi, 1966; Hand, 1975). We will assume in all interpretations of aberrations and exchanges that they occur within the constraint of the translational polarity of the DNA molecule and that at no time must free ends of DNA molecules find each other in order to complete an exchange. This tenet is in general agreement with Revell's exchange theory (1955, 1959, 1974). The process is supported for the chromatin fibril, but is made more complicated by the presence of proteins. Various exchanges will be discussed keeping the intricate structure of the condensed chromosome in mind (Figs. 4, 8, and 27). The light microscopic aspects of aberrations have been treated in a penetrating review by Bloom (1972).

A. Sister Chromatid Exchanges

From the original work of Taylor *et al.* (1957) and of Latt (1973, 1974a,b), it is known that large blocks of chromatin change place in sister chromatids, apparently with great facility. In a previous paper (Bahr, 1975) a mechanism was proposed to explain how one end of a chromatid could undergo exchange with its sister chromatid without changing morphology. In Fig. 28 a mechanism is proposed for the exchange of a block of chromatin somewhere in the middle of a chromosome. On the left side of Fig. 28, a twist has occurred in the daughter strands at two places shortly after replication of the parental duplex. As chromatin fibers gain distance from each other, the twists will come under stress (middle and right side of Fig. 28). Exchange resolves the twist leaving molecules A and B with reciprocal pieces b′ and a′ respectively. The polarity of DNA is in agreement with Brewen and Peacock's work (1969).

Two arguments speak for restricted points along chromatids where crossover can occur. (1) There is a saturation level of $> 1\%$ for the maximum number of sister chromatid exchanges that can occur (Prescott, 1970; Kato and Shimada, 1975; Kato, 1973). Neither X-irradiation nor nucleotide substitution by BUdR (Gatti and Olivieri, 1973; Wolff, 1965; Ikushima and Wolff, 1974) is capable of increasing exchanges beyond the saturation level. Either there are no more exchange sequences per chromatid or the steric distance between reciprocal exchange sequences is too great to be involved in an exchange. Other evidence for restriction is provided in experiments by Trice *et al.* (1975) and Nakagome

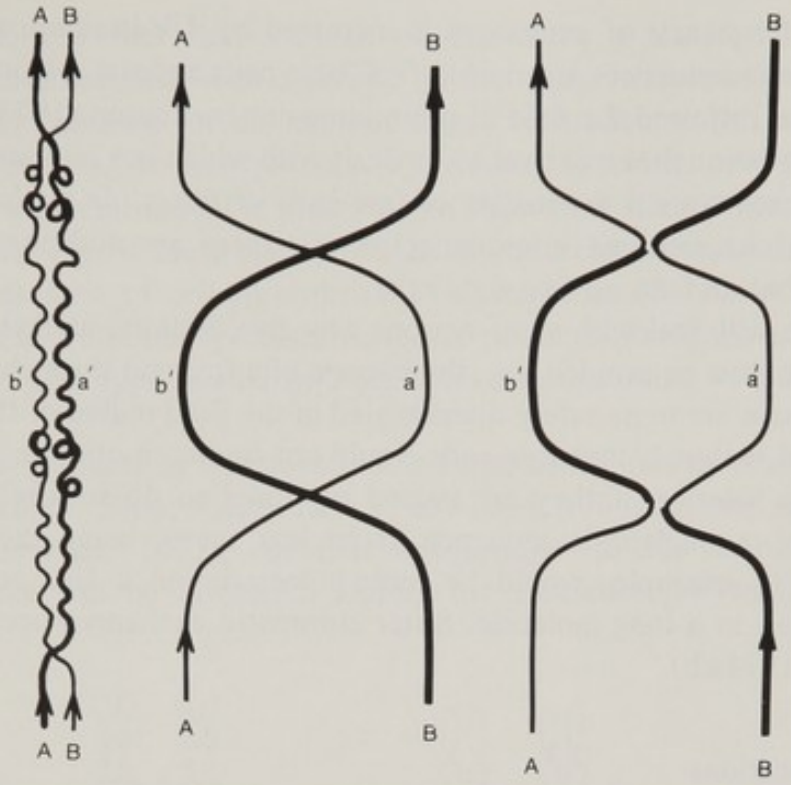


Fig. 28. Proposed mechanism for the reciprocal exchange of a block of chromatin in the middle of a chromosome. See text for explanation. Arrows indicate polarity of fibers.

and Chiyo (1976) using the elegant method of Latt (1974a).* (2) It is difficult to see how the innumerable sister chromatid exchanges that must occur in the course of development and growth of an organism (for the human body this figure may be in excess of 10^{10} for all cells) would proceed with base pair precision, i.e., would occur at exactly matching base pairs at two points in both chromatids. Rather, it is proposed that there are "*exchange sequences*" (Bahr, 1975) that accomplish the task in a smooth and efficient manner, and since these will be short *repetitive* DNA sequences, there is no need for base pair precision. Any unevenness of length resulting from imprecision in selecting the exchange point would be evened out in subsequent exchanges. It will be interesting to see if the frequency of sister chromatid exchanges correlates with any discrete component in the spectrum of repeated DNA sequences, and if the unit of sister chromatid exchange is the same as a crossover unit.

UV-irradiation increases sister chromatid exchange 4–5 times beyond the saturation level when administered during G_1 phase (Kato, 1973; Marin and Prescott, 1964). UV-irradiation produces pyrimidine dimers, which are excised by replication repair processes that are in turn believed to promote exchange.

*See also the very useful variant of Latt's method which utilizes regular Giemsa stain (Wolff and Perry, 1974).

Since the frequency of exchanges is increased by UV-irradiation, the thought that exchange sequences are rich in G–C base pairs appears reasonable. Howard-Flanders has reviewed the field of pyrimidine dimer excision (1973).

One last point that will have to be dealt with which has relevance to all forms of strand exchanges is the matter of free ends of fibers. As we have seen in the section on chromosome interconnectedness, there are additional reasons for assuming that, in fact, no free ends exist.

Here we will deal with some reasons why free ends are unlikely occurrences. True knots must be avoided, i.e., the passage of a free end through a loop. Loops through loops are more safely disentangled in the fluid milieu of the nuclear sap. The second reason is that free ends would not find each other in the vastness of the nuclear interior, if they are indeed supposed to do so after a break. The position of a nucleotide sequence in nuclear space, being the target of an initiator, for example, would be more uncertain on a free end than when incorporated in a long molecule. Sister chromatid exchanges occur during S or G_2 (Latt, 1974a,b).

B. Translocations

The principal elements involved in transferring and intercalating a section of one chromosome to another are those already discussed for sister chromatid exchange. The new aspect is that the insertion or transposition may remain

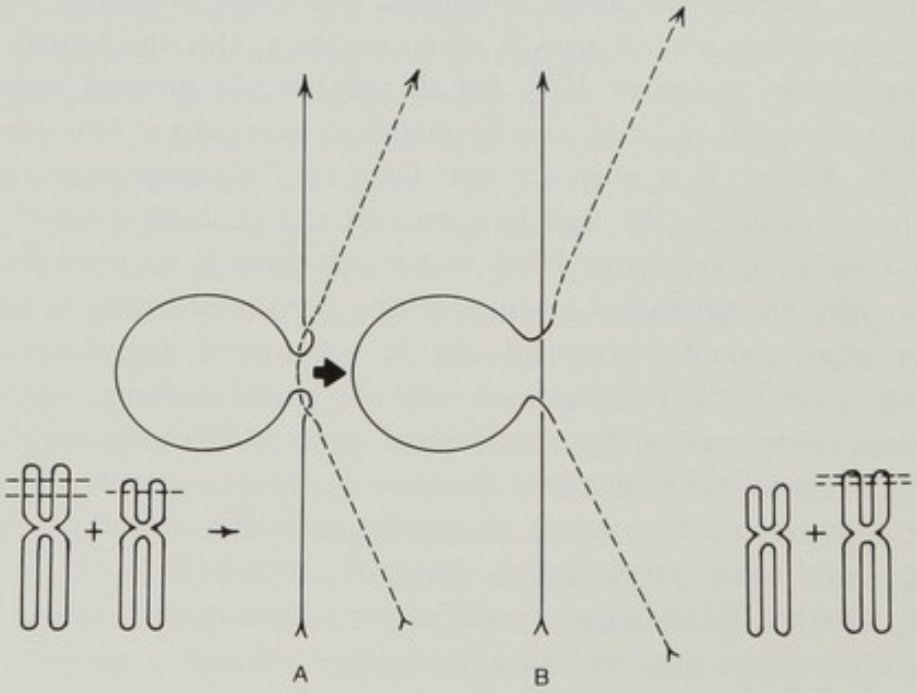


Fig. 29. Transposition or translocation of one piece of chromosome to another chromosome. In A a hypothetical loop formation is depicted. If this formation cannot resolve by sliding apart, one or two exchanges take place in which the loop is transferred to the hatched fiber.

unreciprocated. The fact that a block of chromatin is transferred into the recipient chromosome with such a precise fit that many translocations are only discovered after banding, reveals another severe restriction to this mechanism. Not just any piece can be transposed; *it must have the correct properties to fit*.

In Fig. 29 a general model is presented to show how fiber B incorporates a piece of fiber A. This is illustrated schematically on a chromosome. Interspecies translocation as seen in cell hybridization experiments (Boone *et al.*, 1972) suggests that the molecular species involved at points of exchange are identical or very similar. This type of exchange produces a chromosome with the familiar shape.

Of the many variants of translocation and acrocentric fusions that have been described (Matthey, 1966; Nelson-Rees *et al.*, 1964, 1967; Lithner and Pontén, 1966; Webster *et al.*, 1972), one that was brought to our attention by Dr. Kurt Benirschke will be discussed. During the speciation of *Pinnipedia* two

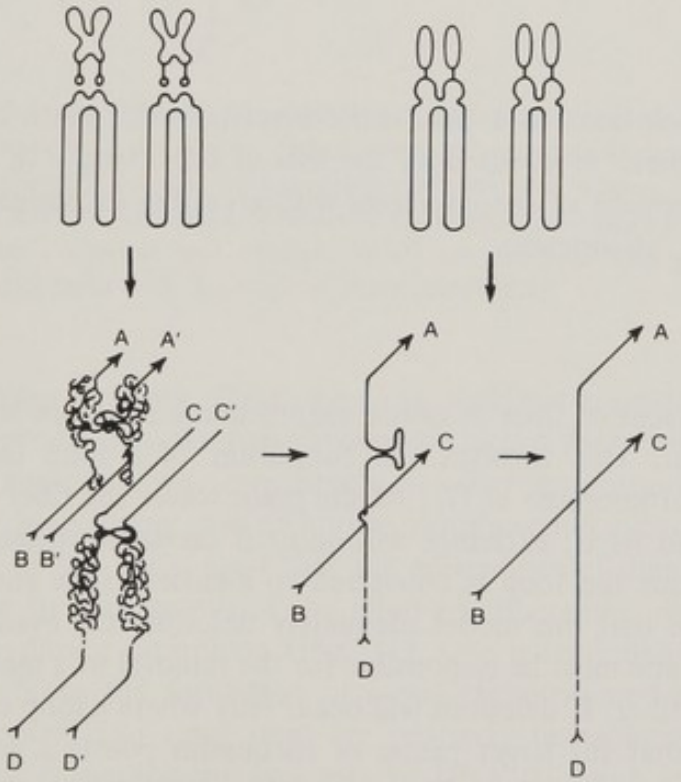


Fig. 30. Acrocentric fusion occurring in pinniped speciation. Satellited chromosomes fuse with acrocentric chromosomes to form submetacentric ones with secondary constrictions in the short arms. These constrictions are the former satellite stalks. The problem is: How does the satellited chromosome lose its centromere? In the lower left, the simplified folded-fiber structure of both chromosomes is shown with afferent B, B′ and D, D′ as well as with efferent fibers A, A′ and C, C′. To the right, the hypothetical exchange between one chromatid fiber of each is shown. BA exchanges with DC. In this exchange, the small centromeric loop(s) of the satellited chromosome is retracted. It is further possible that the specific kinetochore-forming sequence slides to the centromeric level of the acrocentric chromosome.

satellited chromosomes engage in a Robertsonian translocation with two acrocentric chromosomes (Arnason, 1974). The elephant seal and other subspecies have a diploid chromosome number of 34; seals of the tribes *phocini*, including seven recently evolved subspecies, have a diploid chromosome number of 32. The latter having evolved from the primordial chromosome complement through translocation. The fused chromosomes exhibit a prominent secondary constriction in the short arm, which has its origin in the stalk of the satellited chromosomes. In comparing the two karyotypes it is clear that a Robertsonian fusion in the strict sense (fusion of centromeres) has not occurred. If this were the case, the satellite material and the stalks of the satellited chromosomes would not be contained in the fusion chromosomes. These features are clearly recognizable in the fusion chromosomes. Yet only one centromere remains. It is proposed that centromeric loops in the satellited chromosome have been retracted as a consequence of fiber exchange and rearrangement during fusion. This concept is illustrated in Fig. 30. Translocations occur in G_1.

C. Inversions

Two model solutions to a pericentric inversion were given by Bahr (1973a, 1975). One of these proceeds with the loss of some length of fiber; the other involves rearrangement of chromomeres without either exchange or loss of fiber. Inversions are G_1 phenomena.

D. Deletions

Very small pieces of fiber or entire chromosome arms can be deleted by the same mechanism. This involves the formation of a loop in the long fiber constituting a chromosome at G_1. At the point where the fiber touches itself or is twisted around itself, exchange will occur if *exchange sequences* are coming into contact. Thus the loop is converted to a closed circle and lost. Although there is no proof that this model adequately describes the events, some mechanism along this line must be responsible for the removal of a piece from the very long chromatin fiber. If deletions will occur only where a loop can be formed, it is presupposed that for larger pieces of chromatin points a and b are already closely apposed in the nucleus. The nonrandom distribution of deletions among chromosomes is indicative of a relative lack of proximity of points a and b in certain chromosomes. Deletions are G_1 phenomena.

E. Ring Chromosomes

Electron microscopy has shown that a ring chromosome is not an isolated chromosome but is connected to others by fibers (Golomb *et al.*, 1971). Ring chromosomes can be stable configurations and may be found in somatic tissues,

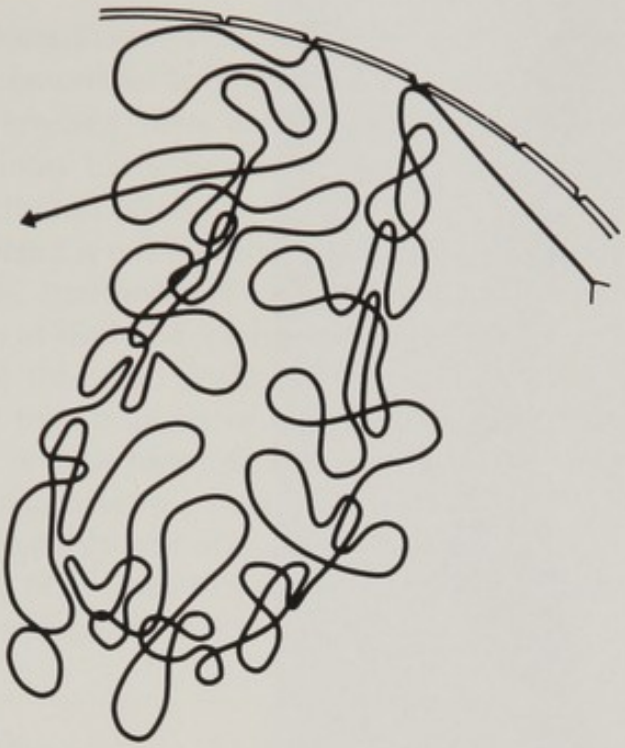


Fig. 31. Schematic drawing of chromosome attachment to the nuclear envelope with fibers extending from both telomeres. Other points of attachment extending from other regions of the chromatid to the nuclear membrane are not shown. This bouquet-type attachment to the nuclear membrane is believed to be the prerequisite for the formation of a ring chromosome. Afferent and efferent fibers are marked. The general concept of attachments is in agreement with the work of Wagenaar (1969).

even those of older patients. Thus, they can undergo normal replication and division, which means that the two chromatids loosen their mutual connecting loops and slide apart in a way comparable to other chromosomes at anaphase. Ring forms have been observed in all human chromosomes except 2, 10, 11, and 12 (Borgaonkar, 1975). We suggest that the appearance of rings throughout the karyotype is due to localization of the telomeric fibers near each other at the nuclear envelope during interphase (Fig. 31). One is reminded of the bouquet state of diptera. Since the formation of a ring chromosome has severe phenotypical consequences, and since rings are relatively unstable aberrations, it is proposed that ring formation involves loss of DNA (Golomb *et al.*, 1971) and exchange between the telomeric ends. Although this exchange is complete, bonds between proteins involved in this exchange may persist and be resolved at a later time.

F. Dicentrics and Multicentrics

Chromosomes exhibit a general tendency to associate, telomere to telomere and telomere to centromere. We have numerous electron micrographs of both

Fig. 32. End-to-end association of four chromosomes. This is not infrequently observed in preparations for electron microscopy. There are pieces of nuclear envelope clinging to the lowest chromosome.

types depicting associations of two to four chromosomes. One of these showing a chain of four chromosomes is reproduced in Fig. 32. Since both centromeres and telomeres are involved, there must be bonding properties common to both. These bonds are most likely protein to protein bonds, between proteins associated with repetitious DNA.

Bonding by proteins is one mode by which temporary dicentrics could appear in a metaphase. The attachment of a chromosome to the centromere of another, however, would most likely be interpreted by light microscopists as an artifact of preparation and therefore would not be recorded. There is no doubt, however, that genuine translocations or end-to-end fusions occur that produce the image of dicentric or even tricentric chromosomes. The event occurs in G_1 and is, in principle, comparable to a translocation. Only one exchange between two telomeric fibers—probably one afferent fiber of one chromosome and the corresponding efferent fiber of another chromosome—is necessary. The result shows up frequently at anaphase.

G. Isochromosomes

D. Borgaonkar (personal communication) notes that the reported and reasonably well-analyzed cases of isochromosomes reveal occurrence only among a limited set of chromosomes in the human karyotype. We interpret this in the following manner. As detailed above, a chromatid is attached to the nuclear envelope with fibers extending from both telomeres. Imagine the two fibers of a recently replicated metacentric or submetacentric chromatid arching in a horseshoelike loop in the interior of the nucleus. At or near its apogee the complex exchange takes place which links two q and two p arms to each other (Figs. 16 and 17; Bahr, 1975). This exchange is constrained by the transcriptional polarity of the fibers involved. Only those chromosomes that possess fibers with a respective spatial relationship that would lend itself to this type of exchange will be involved in isochromatid formation. We may have, in this case, another instance in which a specific configuration and proximity of fibers at interphase is revealed.

H. Triradials and Quadriradials

It is not possible, at this time, to explain the formation of triradials except that they appear to be the result of selective endoreduplication. When such chromosomes are G-banded, all three components appear to be homologous.

The case of quadriradials (Qr) is somewhat clearer. According to German (1974) the majority of Qr are formed by the joining of two homologues in one arm. The frequency of Qr formation is usually low, but in Bloom's syndrome it is quite significant, 0.5–14% (German, 1969). Here an increased number of cells

with distorted nuclear outline and micronuclei are also seen (German and Crippa, 1966). As discussed above (see Section IV,D), the telomeres of an interphase chromosome, and the telomeres of the homologous chromosome are anchored relatively close to each other on the nuclear membrane. In the normal course of events innumerable interdigitating loops between the chromosomes have to be disentangled. An electron micrograph of late prophase in a normal lymphocyte gives some idea of the complexity of this process (Fig. 20). When these anchoring points are brought close together by an abnormal process, such as the distortion of the nuclear envelope described for Bloom's syndrome, the distal parts of the chromosome behave as if they are in meiosis. In all Qr that have been found in the literature, the distal ends of chromosomes are responsible for the contact. Qr dissolve cleanly into the constituent chromosomes of anaphase. There is no evidence in published articles that Qr produce anaphase bridges. In many instances, however, dicentrics occur in the same metaphase. If indeed the peculiar wrinkling of the nuclear membrane is the reason for Qr formation, then this feature is likely to appear in daughter cells after division because the reasons for wrinkled nuclei persist. German's evaluation of 101 Qr in 18 patients (1974) implies, in the sense of this concept, that anchor points of some telomeres are closer to each other than others. Some chromosomes in this relatively large sample show significantly higher frequency of Qr than others; 17 were observed for chromosome No. 1, while none were seen for No. 2.

I. Gaps and Breaks

The chromosomal aberrations discussed previously are readily induced by ionizing radiation, chemicals, and viruses. These agents also produce chromatid and chromosome gaps and breaks. A gap has traditionally been defined as an interruption in a chromatid. The size of the interruption should not exceed the width of a chromatid. If the interruption is found to be larger, it is called a break.

Comings (1974) has given an extensive treatment of the subject. This article will therefore limit itself to observations, measurements, and deductions made in the author's laboratory. It has been found that all of the commonly described gaps and breaks clearly show chromatin fibers bridging the interruption. The number of bridging fibers varies from 2 to 8, clearly a decrease from the normal 8 to 15 fibers. These residual fibers provide the reason why chromatid pieces are generally found near the chromosome from which they were broken. Interdigitating loops from unaffected sister chromatids also play a role in maintaining the position of chromatids containing gaps or breaks. In 1970 Scheid and Traut, and Brinkley and Shaw, studied X-irradiated, formalin-fixed chromosomes of *Vicia faba* by light and scanning electron microscopy. They found that gaps were bridged by material that was detectable in the scanning electron microscope and

by ultraviolet microscopy, suggesting that the material contained nucleic acid. Brøgger (1974) made comparable observations on apparently spontaneously damaged chromosomes. The chromatid with a break was found to be, on the average, 8.9% shorter than its sister chromatid. It is known that chromosome No. 3 shows a high frequency of spontaneous damage in regions p21 (Lubs and Samuelson, 1967), and p22-32 (Brøgger, 1971).

The next question to be asked is whether the continuity of the mononeme is interrupted by the gaps and breaks. Comings (1974) has answered in the affirmative. He views the bridging fibers as representing discontinuous stretches of fiber that are connected by protein bonds to the fibrous structure of the main body of the chromatid. Brøgger (1975), in contrast, considers the major effect of ionizing radiation to be on proteins and thinks that conformational changes in tertiary protein structure could lead to gaps and the so-called breaks.

On the grounds that a decreased number of fibers are found in the interruption, we feel in agreement with Comings (1974), that a break and ensuing rearrangement of fibers might have occurred. Nonetheless Brøgger's ideas about proteins deserve careful scrutiny. Too much attention has been paid to the effects of ionizing events on DNA in irradiated prokaryotes. The eukaryotic chromatin, because of the presence of large amounts of structural protein, may prove to be an entirely different matter.

On the assertive side of this problem, it can be reported that in the majority of cases there is no measurable loss of mass from a chromosome with either a gap or a break. This has been demonstrated by quantitative electron microscopy of 30 lesions, where the mass of chromatids with gaps or breaks was compared to the mass of the unaffected sister chromatid. These measurements are not in conflict with either Comings' or Brøgger's interpretations. Only when it is possible to trace a single fiber in a chromatid may the question of gaps and breaks be solved.

VII. MATERIALS AND METHODS

We have consistently used the following method for the preparation and visualization of chromatin at interphase and metaphase. PHA-stimulated lymphocytes or cells from tissue culture are first exposed to hypotonic Hanks' solution (one part distilled water to one part standard Hanks). If cells prove to be resistant to swelling, as cells in tissue culture often are, distilled water alone may be used. In either instance 10 minutes of exposure of the cells is usually sufficient. The number of well-spread chromosomes or nuclei under the electron microscope determines whether the swelling period needs to be prolonged or shortened. Swollen cells are centrifuged for 5 minutes at 100 g and the supernatant carefully decanted. Part of the pellet is then pipetted onto a stainless steel

spatula and applied to the water surface as illustrated and described in detail by Bahr (1973a). Spread chromatin is picked up on Formvar-coated grids for electron microscopy, passed through a graded series of ethanol and acetone or acetone alone, and critical-point dried according to the method of Anderson (1951).

Prepared grids were stored over a desiccant and eventually viewed in an electron microscope. An accelerating potential of 100 kV was used throughout with an objective aperture 100 μm (2×10^{-2} steradians) and a condenser aperture 100 μm in diameter. The film was exposed to yield a limited range of background transmission—between 0.17 and 0.35—over the background fog. Such electron micrographs can be evaluated for integrated transmission in an object and its background, rendering an equivalent value for the dry mass of the object. Standardizing the procedure converts relative mass to absolute mass. The error of the method is $\mp 2\%$ for relative determinations and $\mp 9\%$ for absolute mass calculations (Zeitler and Bahr, 1962; Bahr and Zeitler, 1965). A detailed description of the step-by-step procedures for determining quantitative mass by electron microscopy has been given by Bahr (1973b), where various means for photometric evaluation of contrast are discussed. Stereo pairs were electrographed using the top entry goniometer stage of a Hitachi HU-12 electron microscope.

ACKNOWELDGMENTS

Most of the electron micrographs were taken by my collaborator Walter F. Engler, who has assisted me for the last 15 years with the electron microscope. The manuscript was critically read and edited by Dr. Robert Shoemaker, to whom I am indebted. Last but not least, I am grateful to Mrs. Jeanette Schwartz, who in spite of illness, typed the manuscript.

This work was supported in part by grant No. R01 GM 20046-01 from the National Institutes of Health, PHS, DHEW, and grant No. 1-397 from The National Foundation-March of Dimes.

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6

Premature Chromosome Condensation and the Fine Structure of Chromosomes

POTU N. RAO

I. INTRODUCTION

The fusion between a mitotic and an interphase cell mediated through UV-inactivated Sendai virus usually results in the transformation of the interphase nucleus into discrete chromosomes under the influence of mitotic factors. The morphology of the chromosomes resulting from the premature condensation of the interphase nucleus depends upon the position of the interphase cell in the cell cycle at the time of fusion. For example, a cell in G_1

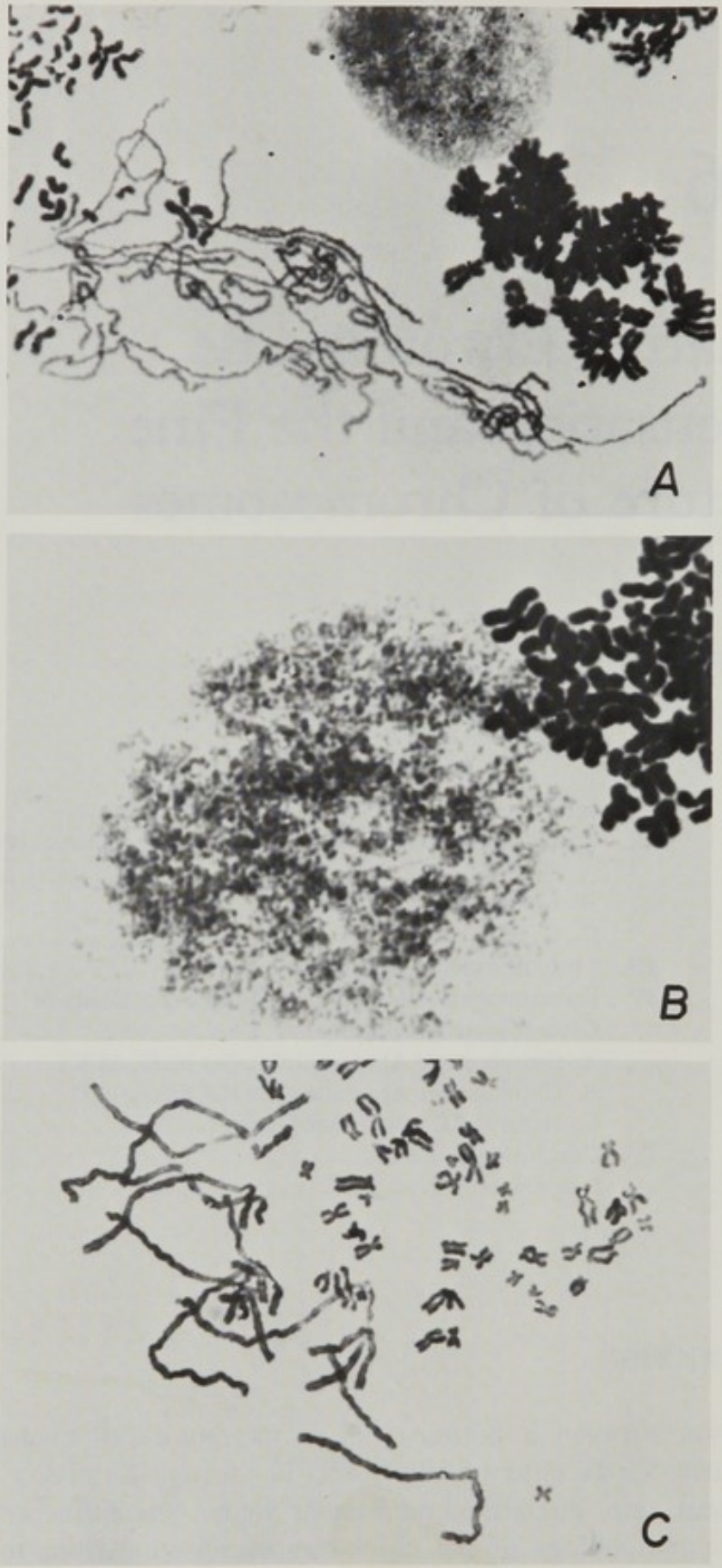
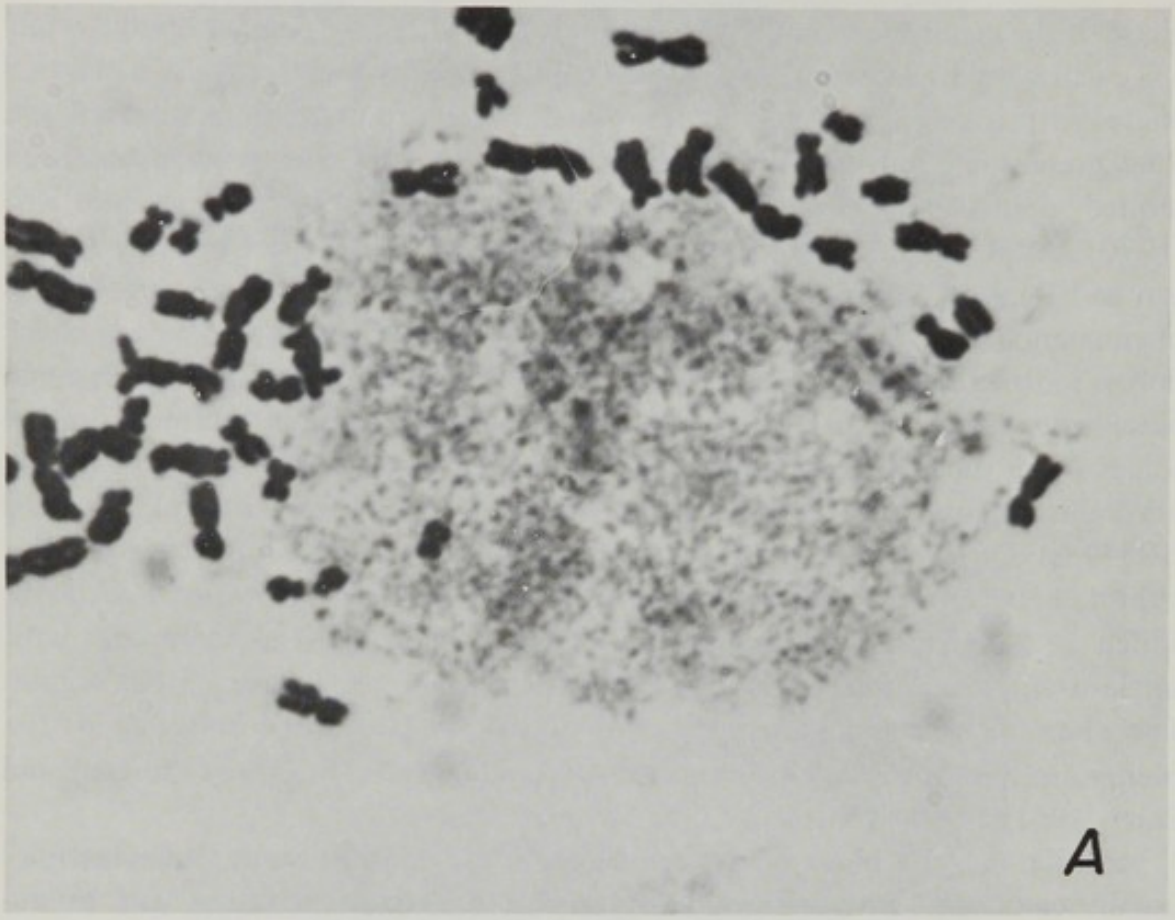


Fig. 1. Prematurely condensed chromosomes of rat kangaroo cells (*Potorous tridactylis*) cell line (pt K_2) after fusion with mitotic HeLa cells. (A) G_1 PCC with single chromatids; (B) S PCC with pulverized appearance; (C) G_2 PCC with two chromatids closely attached to each other. From Sperling and Rao (1974a).

phase of the cell cycle would yield chromosomes with only one chromatid, while a G_2 cell would produce chromosomes consisting of two chromatids. A cell in S phase, surprisingly, produces chromosomes which are unevenly condensed and thus present a "pulverized" appearance (Fig. 1). This phenomenon has been termed premature chromosome condensation (PCC) by Johnson and Rao (1970). Although this phenomenon has been recognized rather recently (Johnson and Rao, 1970), some of the observations that fall within the scope of this phenomenon were made earlier. In 1930 while Bleier was studying meiosis in the pollen mother cells of *Triticum* × *Secale* hybrids, he observed in the occasional binucleate pollen mother cells that one nucleus had distinctly normal chromosomes while the other contained long and slender threads without a recognizable nucleolus (Bleier, 1930). Similar findings were reported by Kihara and Lilienfeld (1934) on *Triticum* × *Aegilops* hybrids and by Holden and Mota (1956) on the hybrids between *Avena barbata* and *Avena strigosa*. In *Haemanthus* endosperm, which is a syncytium, Östergren and Bajer (1961) observed some cells with single-stranded chromosomes together with normal metaphase chromosomes. The aberrant chromosomes reported by these investigators correspond to the prematurely condensed chromosomes of the G_1 cells. In fact, Östergren and Bajer (1961) assumed this to be the case.

Another widely observed phenomenon is the "chromosome pulverization" usually associated with multinucleate cells formed after infection with myxoviruses. Originally it was assumed that this massive fragmentation or pulverization of chromosomes was the direct result of viral infection (Nichols *et al.*, 1964, 1965, 1967). Pulverization is generally associated only with those viruses that can induce cell fusion, such as the NDV group (measles, Sendai, and mumps) of myxoviruses (Waterson, 1962; Roizman, 1962), herpes (Benyesh-Melnick *et al.*, 1964) and adenoviruses (Roizman, 1962). For example, influenza, a myxovirus that does not cause cell fusion does not induce chromosome pulverization (Cantell *et al.*, 1966). In a number of cell fusion studies chromosome pulverization was observed in multinucleate cells formed after exposure to inactivated Sendai virus (Sandberg *et al.*, 1966; Kato and Sandberg, 1967, 1968a,b,c; Takagi *et al.*, 1969; Stenman and Saksela, 1969). These observations clearly indicate that the infectivity of virus is not essential for the production of multinucleate cells or the induction of chromosome pulverization. Pulverization was also observed in the syncytia of *Physarum polycephalum* where no virus was involved (Guttes and Guttes, 1963; Rusch *et al.*, 1966). Although pulverization is often associated with a multinucleate condition, it has also been reported in mononucleate cells where only some of the chromosomes, usually the late replicating ones, are affected (ZurHausen, 1967; Miles and O'Neill, 1969). In the light of the phenomenon of premature chromosome condensation all these observations can be explained as follows: (1) The "pulverized chromosomes" are in fact the prematurely condensed chromosomes of the S phase nucleus (Fig. 2). As will be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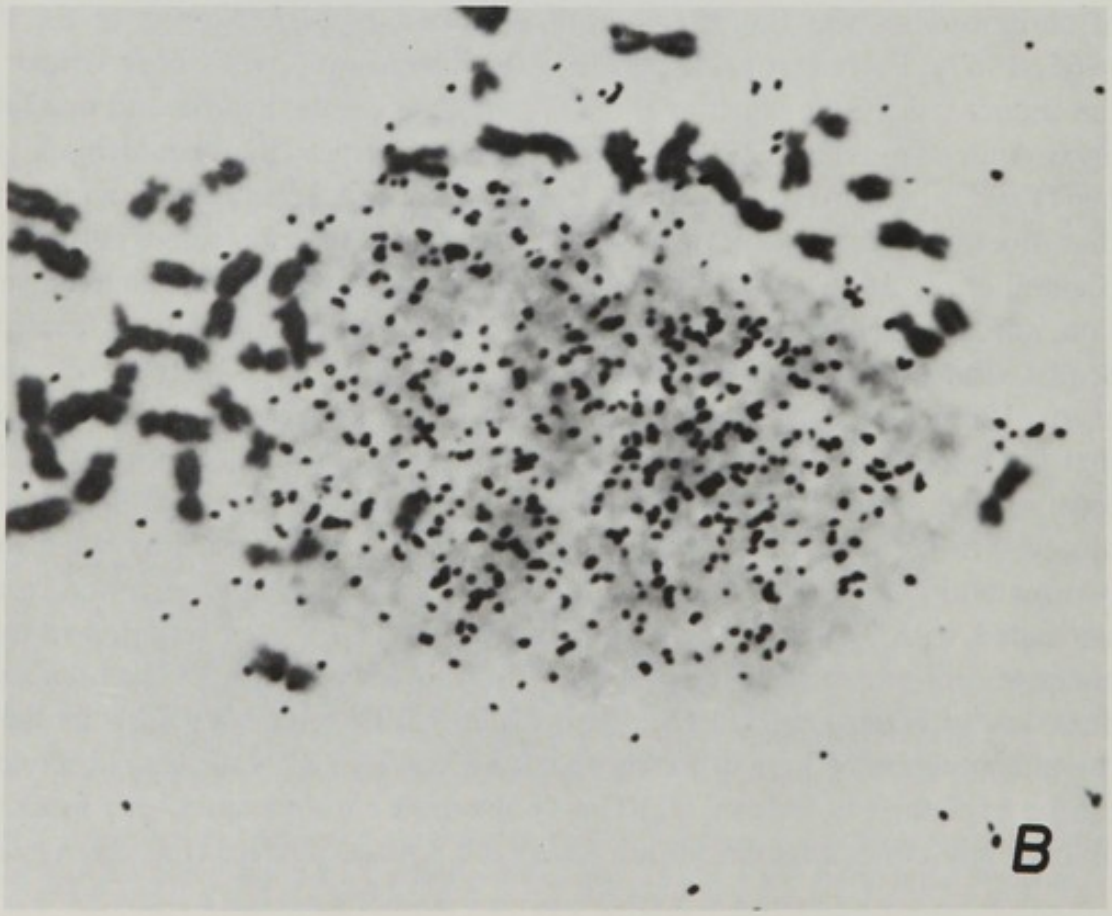
B

shown later these chromosomes are not really fragmented into discontinuous pieces as the term "pulverization" implies. (2) The premature chromosome condensation occurs due to mitotic asynchrony between the nuclei of a bi- or multinucleate cell. The advanced nucleus which enters mitosis earlier than the other induces PCC in the lagging nucleus (Johnson and Rao, 1970). This is best illustrated by fusing synchronized populations of mitotic with S phase cells.

The phenomenon of PCC has been the subject of several reviews (Rao and Johnson, 1972, 1974, Sperling and Rao, 1974a). The present chapter deals primarily with the contribution of this phenomenon to the knowledge of the fine structure and organization of mammalian chromosomes.

II. STRUCTURE OF PREMATURELY CONDENSED CHROMOSOMES (PCC)

A. G_1 PCC

From Fig. 1 it is obvious that the structure of the prematurely condensed chromosomes reflects the state of chromatin at different phases in the cell cycle. The G_1 PCC, like the chromosomes in the preceding anaphase, consist of single chromatids indicating the unreplicated state of the genome. The centromere region of G_1 PCC, which is not detectable in standard preparations, can be made distinctly visible by the C-banding technique (Unakul *et al.*, 1973). It is also possible to induce G banding in G_1 PCC (Fig. 3). A closer examination of the structure of G_1 PCC may reveal more about the conformational changes taking place in the chromatin. In general, the amount of electron microscope work done with PCC is rather limited. Hence, most of our information comes from light microscope studies. Recently, Schor *et al.* (1975) made a detailed study of the changes in the morphology of PCC at different points during the G_1 phase. They also studied the effects of UV irradiation on the structure of G_1 PCC. These studies revealed a progressive change in the morphology of the G_1 PCC as cells progressed towards the DNA synthetic period (Fig. 4). They reported that in HeLa cells at 2 hours after the reversal of the mitotic block, which was considered to be the beginning of G_1, approximately 90% of the G_1 PCC were relatively short and well condensed (Fig. 4A). In a sample taken 5 hours later, almost all of the G_1 PCC were significantly longer and more attenuated (Fig. 4B). The total contour length of the PCC from the cells at the end of G_1 period

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Fig. 2. S PCC, the product of fusion between an S phase cell of *Microtus agrestis* and a HeLa cell in mitosis. The *Microtus* cells were pulse labeled for 15 minutes with ^{3}H-TdR just before fusion. (A) S PCC exhibiting pulverized appearance. The darkly stained chromosomes are of mitotic HeLa cells. (B) A radioautograph of the same cell with silver grains indicating that this cell was in S phase at the time of pulse labeling and subsequent fusion.

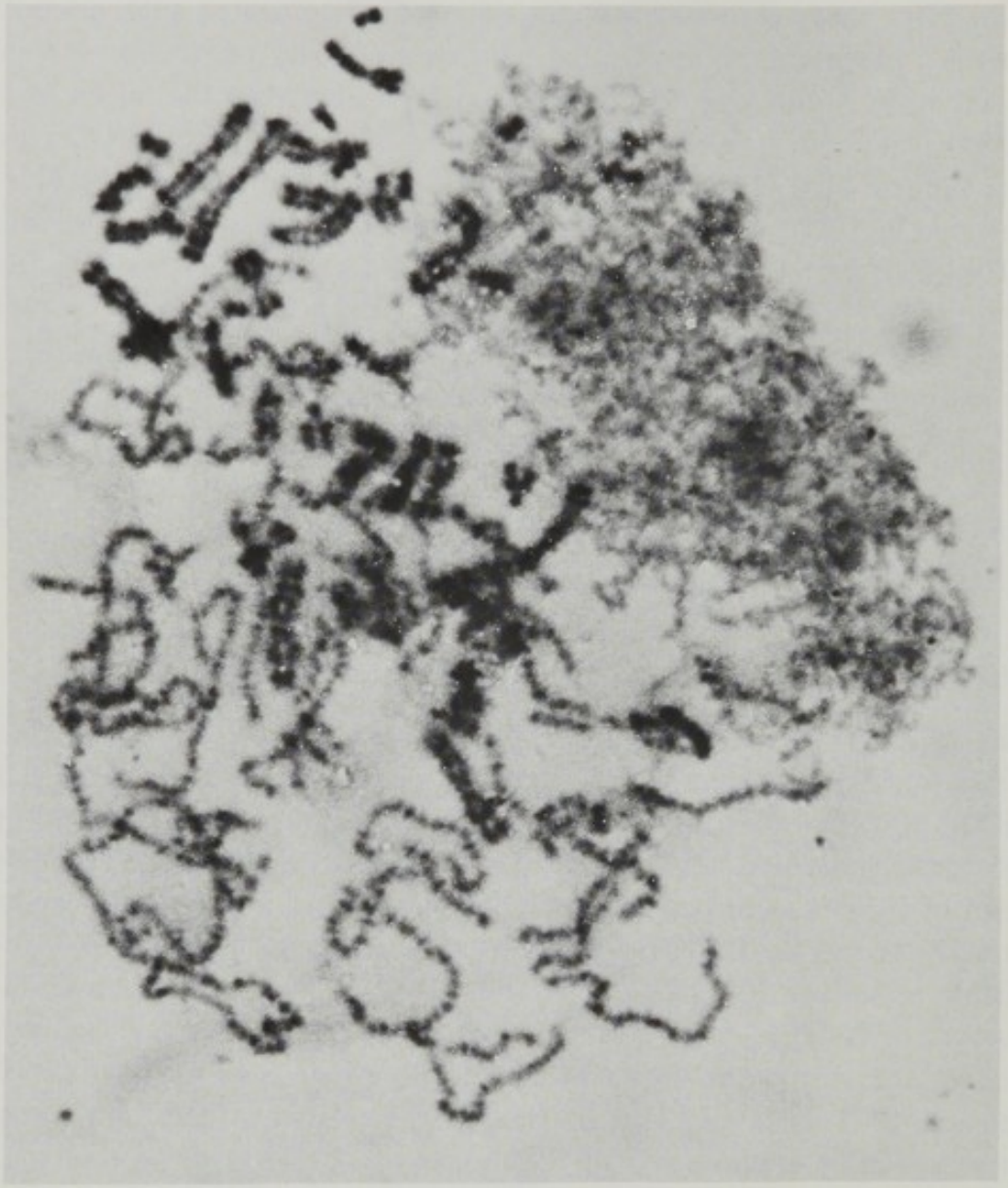

Fig. 3. Giemsa banding in the G_1 PCC of Chinese hamster lung cells (4,300X). Courtesy of M. Appley and A. Zona, State University of New York, Brockport.

was twice than that from early G_1 cells. These data indicate that the decondensation of chromosomes initiated during the telophase of mitosis continues throughout the G_1 period. The authors also noted that the response of G_1 cells to UV irradiation as measured by the degree of attenuation or decondensation of the PCC increased as they progressed towards S phase. For example, early and late G_1 HeLa cells irradiated with UV light (160 J m^{-2}) before cell fusion produced PCC which were greatly decondensed as compared to the controls (compare Figs. 4A and B with Figs. 4C and D). This suggests that the conformational changes of chromatin occurring during G_1 make them progressively more sensitive to the effects of UV irradiation (Schor *et al.*, 1975).

The process of UV-induced unscheduled DNA synthesis which involves the excision of the damaged regions of the DNA molecule and subsequent synthesis

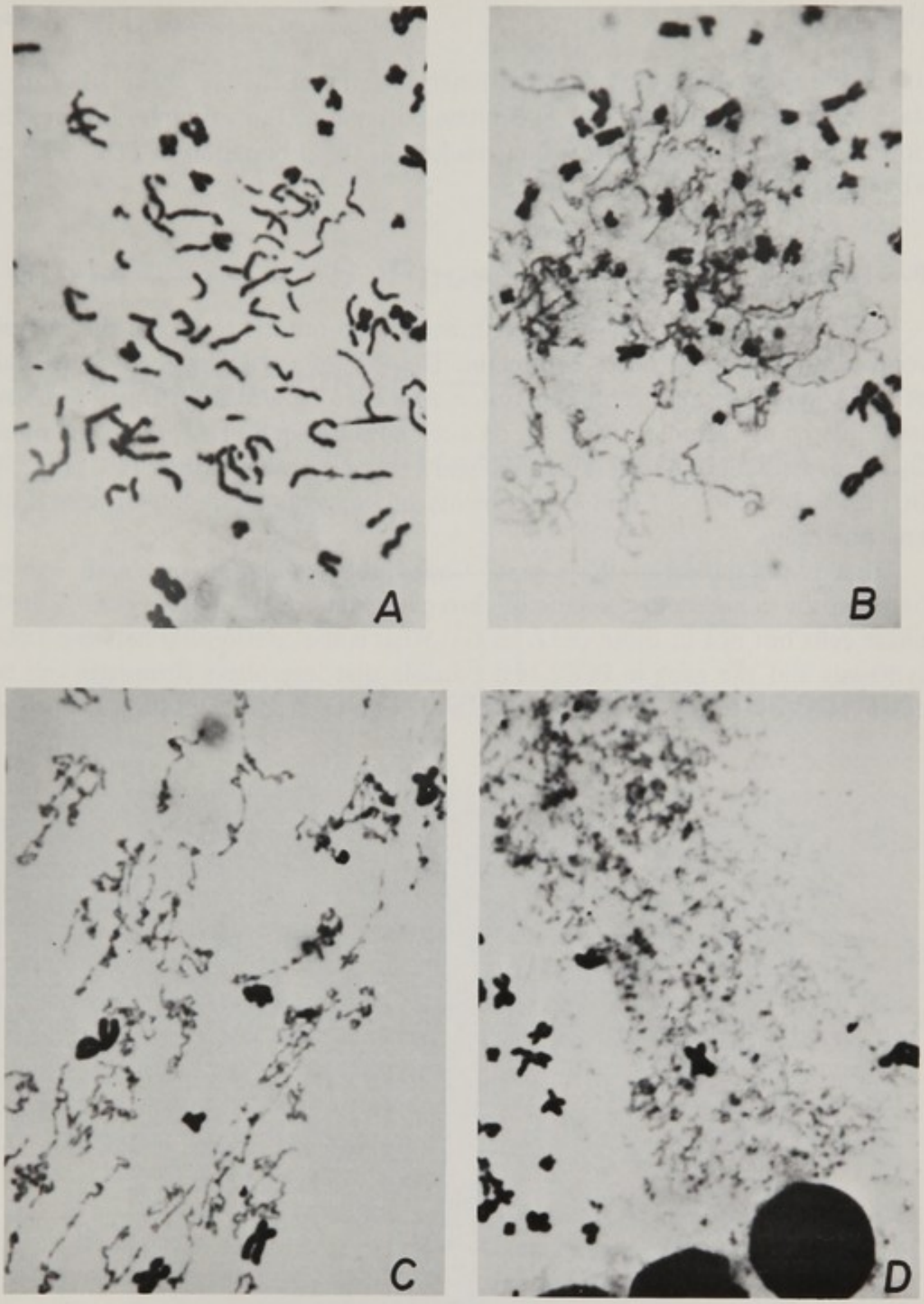

Fig. 4. Changes in the degree of condensation of PCC of HeLa cells during G_1 phase. (A) PCC showing the condensed morphology typical of cells at the beginning of G_1 (t=0 hours). (B) Relatively more decondensed PCC of cells later in G_1 (t=5 hours). (C) Attenuated G_1 PCC from HeLa cells exposed to UV irradiation (160 J m^{-2}) at the beginning of G_1 and fused with a mitotic cell 30 minutes later. This preparation resembles PCC from unirradiated cell at the end of G_1. (D) Highly attenuated (S-like) PCC from HeLa cell exposed to UV-irradiation (160 J m^{-2}) in late G_1 (t=5 hours) and fused with a mitotic cell 30 minutes later. (From Schor *et al.*, 1975.)

to fill the gaps alters the conformational patterns of the G_1 chromatin into a state somewhat similar to that of S phase chromatin. For example, UV irradiation of late G_1 cells prior to fusion resulted in the production of PCC, 90% of which were S-like (Schor *et al.*, 1975).

B. S PCC

The fusion of an S phase cell with another in mitosis results in the uneven condensation of the S phase chromatin. The PCC of a cell which was at its peak of DNA synthesis at the time of fusion appear to be a mass of finely "pulverized" pieces of chromatin (Fig. 2). As a cell advances in S phase, more and more G_2 elements with double chromatid regions become visible in their PCC (Fig. 5). In Fig. 5, both the G_1 and G_2 segments of the genome are interspersed with unstained gaps.

What is the nature of these gaps? Gaps, probably the uncondensed regions between the condensed segments of chromatin, are present only in PCC of the S phase cells but not in those of G_1 or G_2. What is the relationship between DNA synthesis and the gaps in PCC? Is it possible that interphase chromatin can be condensed (by fusion with mitotic cells) before or after replication but not at

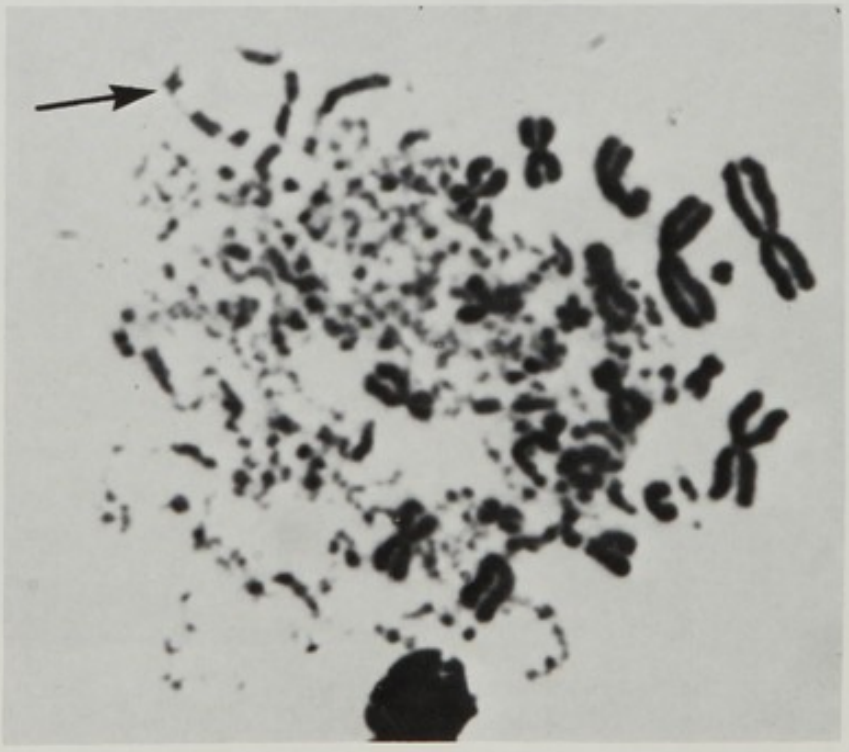

Fig. 5. Premature chromosome condensation of an S-phase nucleus in a M/S fused cell. The arrow points to a chromosome in which some regions were replicated; other regions were not. Replicated regions appear thick and heavily stained. (From Rao and Johnson, 1974.)

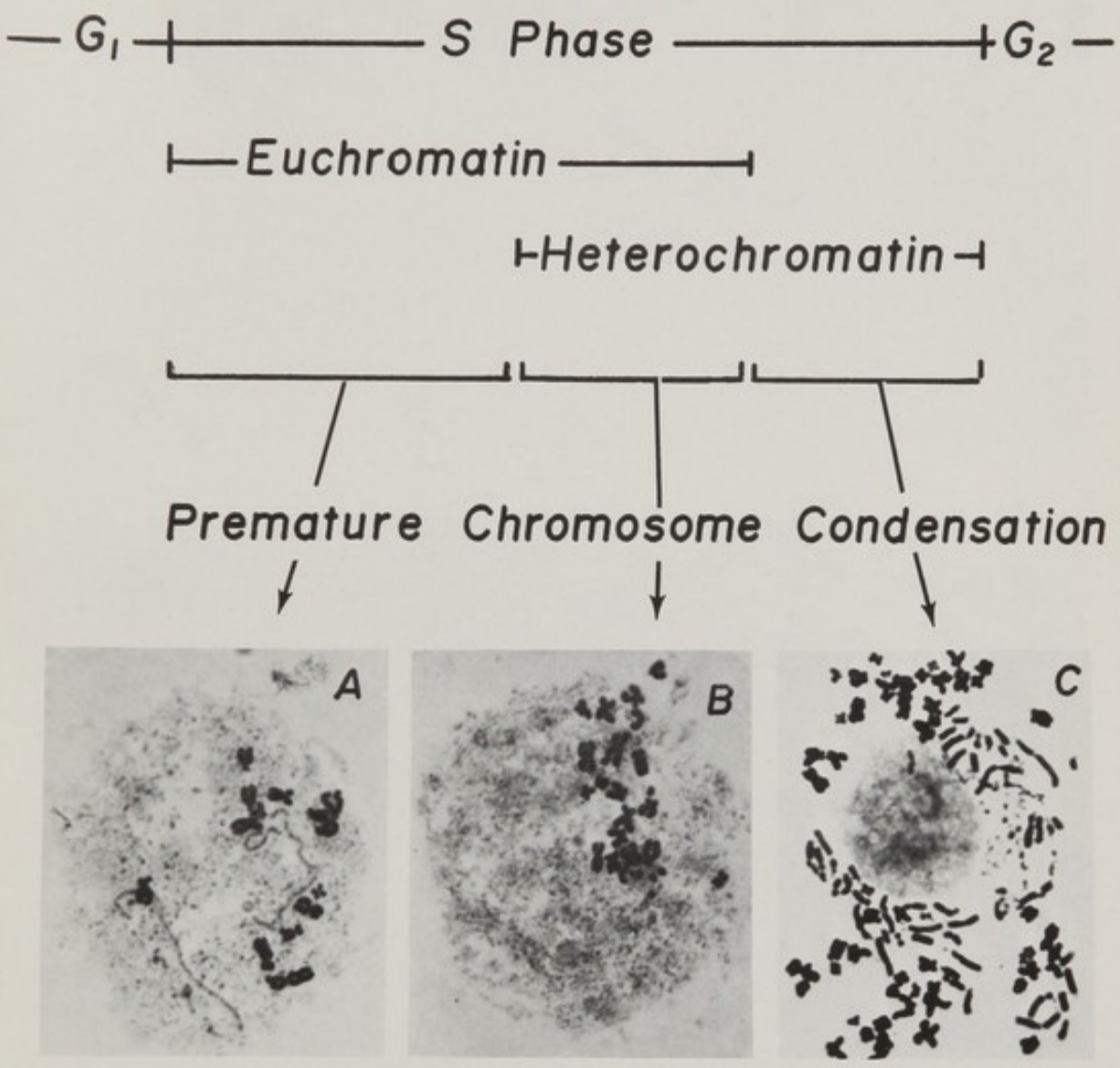

Fig. 6. Prematurely condensed chromosomes of *Microtus agrestis* at three consecutive stages of S phase. A random population of *M. agrestis* cells were fused with synchronized mitotic HeLa cells. The highly condensed and darkly stained chromosomes are of mitotic HeLa cells. (A) Early S: The autosomes of the *Microtus* cell present a pulverized appearance. The heterochromatic regions of the two X chromosomes are in G_1 phase as indicated by their single chromatid structure. (B) Mid S: All chromosomes, both autosomes and X chromosomes, of thre *Microtus* cell are "pulverized." (C) Late S: The autosomes of the *Microtus* cell are double-stranded G_2 chromosomes and the heterochromatic regions of the two X chromosomes still appear to be pulverized. (From Sperling and Rao, 1974b.)

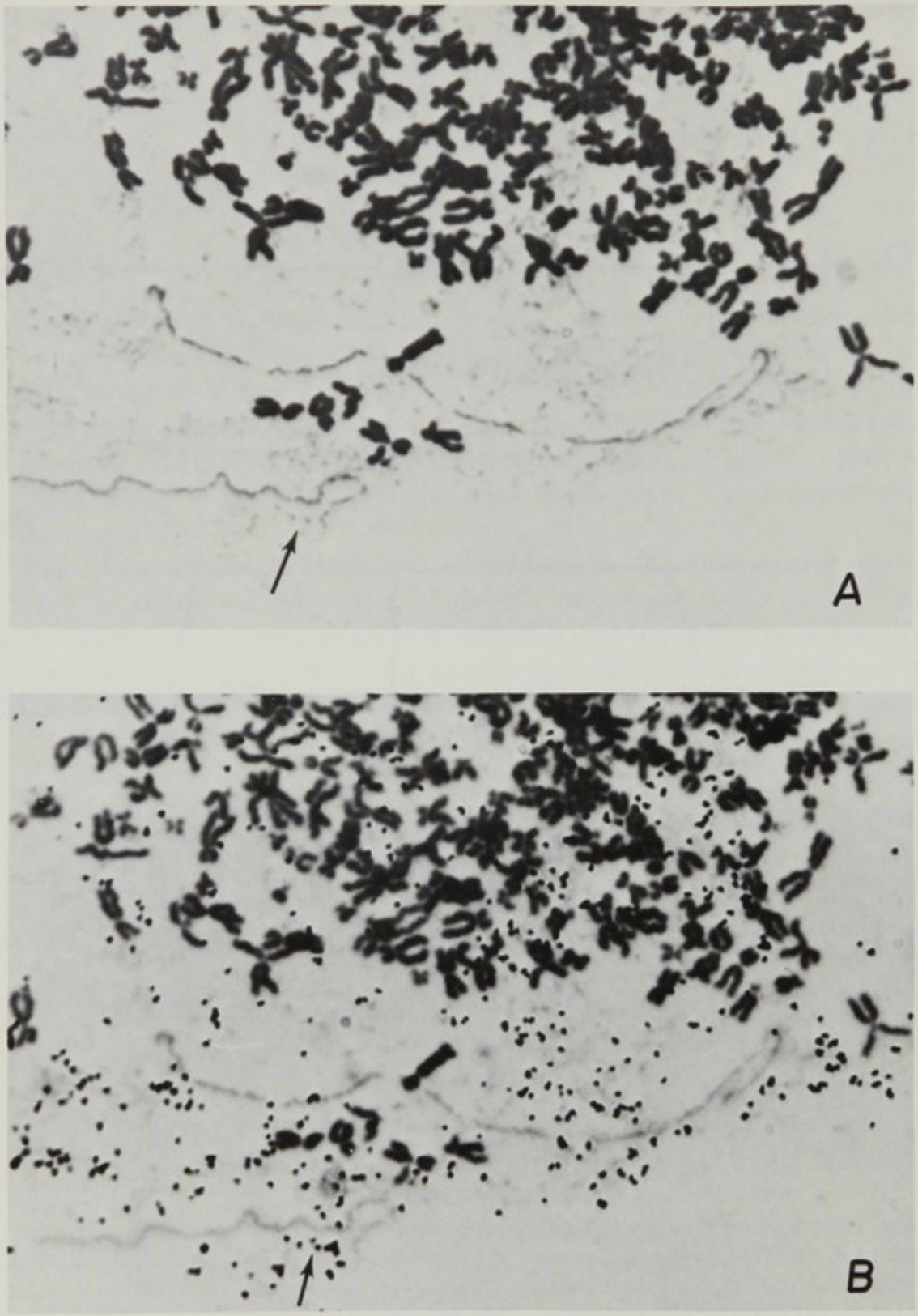

Fig. 7. Localization of replication sites in the prematurely condensed chromosomes. (A) Mitotic HeLa cells were fused with *Microtus* cells in interphase. The arrow points to the euchromatic region of an X chromosome which is in the process of DNA replication. This region resembles a chain of beads, i.e., gaps alternating with condensed segments. (B) A radioautograph of the same cell. The arrow points to the silver grains, and their location corresponds to the gaps of the above figure. Thus, the gaps in the S PCC appear to be the exact site of replication or a replicon.

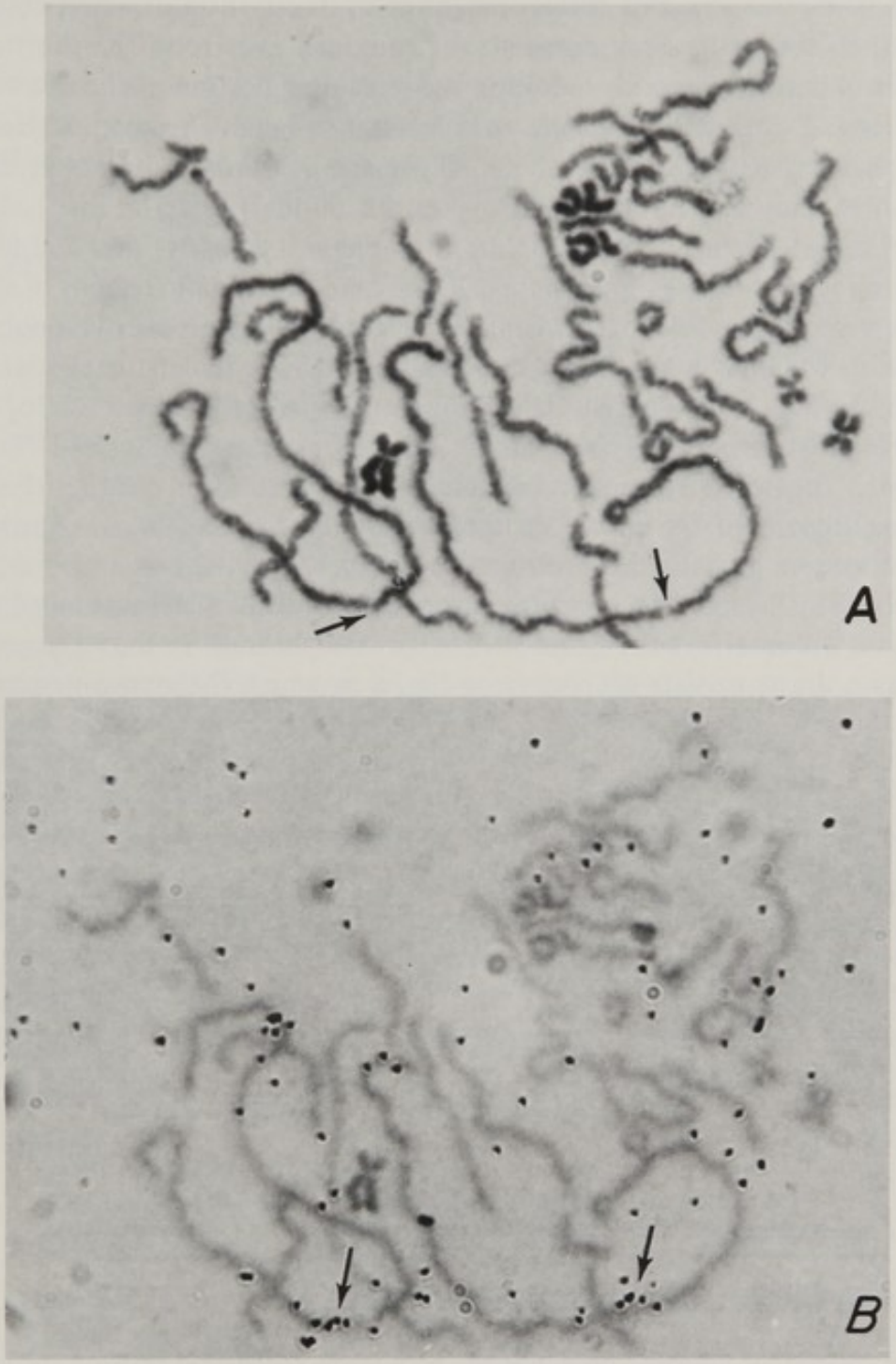

Fig. 8. (A) PCC of a *M. agrestis* cell in late G_2 phase. (B) A radioautograph of the same cell. Note that label is localized in the centromeric regions (arrows) of the X chromosomes, which indicates that these regions are the last to replicate. (From Sperling and Rao, 1974b.)

the point of replication? A satisfactory answer to this question has been found by studying the PCC of the cultured cells of the European field vole, *Microtus agrestis*. The female of this species has most of its constitutive heterochromatin localized in the entire long arm and the proximal quarter of the short arm of both the X chromosomes. In addition the remaining portion of the short arm of one of two X chromosomes undergoes facultative heterochromatinization. The late replicating nature of these heterochromatic X chromosomes and the ease with which they can be identified makes this an ideal material for study. The PCC of three cells at different points in S phase, i.e., early, mid and late, are shown in Fig. 6. In the early S phase cell, the heterochromatic regions of the two X chromosomes are in G_1 state while all the autosomes appear to be pulverized because their DNA is in a replicative phase. A closer look at a similar cell is provided in Fig. 7. In this cell the euchromatic region of the short arm of one of the two X chromosomes (shown by arrows) appears "pulverized" but the condensed fragments resemble the arrangement of beads on a string (Fig. 6A). A radioautogram of the same cell shows that the location of the black silver grains corresponds with the position of gaps between the condensed segments (Fig. 6B). This leads us to conclude that the uncondensed and unstained gaps in the S PCC are in effect the exact sites of DNA replication or the replicons. This conclusion draws further support from Fig. 8 in which the centromeric regions

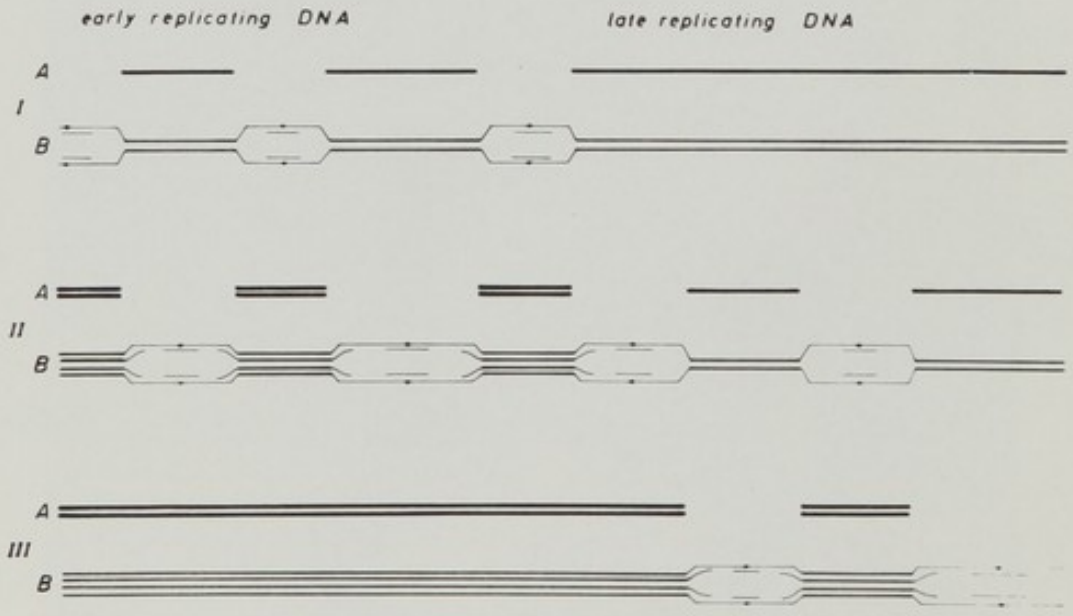

Fig. 9. Schematic drawing concerning the presumable correlation between the morphological appearance of PCC at early (I), mid (II), and late (III) S phase and the molecular model of chromosomal DNA replication of Huberman and Riggs (1968). (A) Schematic drawing of PCC: G_1 regions are single, G_2 regions are double line; the sites of DNA replication are the open gaps. (B) Model of chromosomal DNA replication according to Huberman and Riggs (1968). Synthesis proceeds in both directions from each site of initiation. (From Sperling and Rao, 1974a.)

of the two X chromosomes (shown by arrows) appear to be practically the last segments of DNA to be replicated (Sperling and Rao, 1974b).

These observations on the morphology of the PCC can be explained with the help of a molecular model proposed by Huberman and Riggs (1968) for DNA synthesis in eukaryotic cells. In Fig. 9 an attempt has been made to correlate the molecular events of DNA replication with the morphological picture of the prematurely condensed chromosomes.

C. G_2 PCC

There is a great similarity between G_2 PCC and mitotic chromosomes during prophase except that the former are more extended and longer than the latter. The G_2 PCC consist of two chromatids which are generally attached to each other. The extended nature of G_2 PCC may reflect the conformational state of chromatin at that point in cell cycle. To answer the question whether there was a progressive condensation of chromatin during the G_2 period, Sperling and Rao (1974b) measured the long arm of the prematurely condensed heterochromatic X chromosome in *Microtus agrestis* cells at different points in G_2. Table I shows that the process of chromosome condensation probably initiated soon after replication proceeds throughout G_2 and reaches the maximum by metaphase. These observations serve as a visual evidence for the existence of chromosome condensation cycle that is tightly coupled with the division cycle in mammalian cells (Mazia, 1963; Pederson and Robbins, 1972; Pederson, 1972).

Initial attempts to band G_1 and G_2 PCC with quinacrine mustard revealed that Q-banding could be induced in both these types, but it was more distinct in the latter (Patil *et al.*, 1972). Subsequently, C and G banding was induced in the PCC of HeLa cells. The G_2 PCC being longer than prometaphase and metaphase chromosomes, the Giemsa banding of G_2 PCC gave more detailed delineation of chromosomes. In general, a segment containing two or three bands in metaphase

TABLE I
Relative Lengths of X Chromosomes of *Microtus agrestis* in G_2 and Mitosis[a]

Phase of cell cycle	Number of chromosomes scored	Average length (μm)	Relative length
Early G_2	3	48.0	4.2
Mid G_2	13	39.6	3.5
Late G_2	4	16.4	1.5
Metaphase	20	11.3	1.0

[a] From Sperling and Rao (1974b).

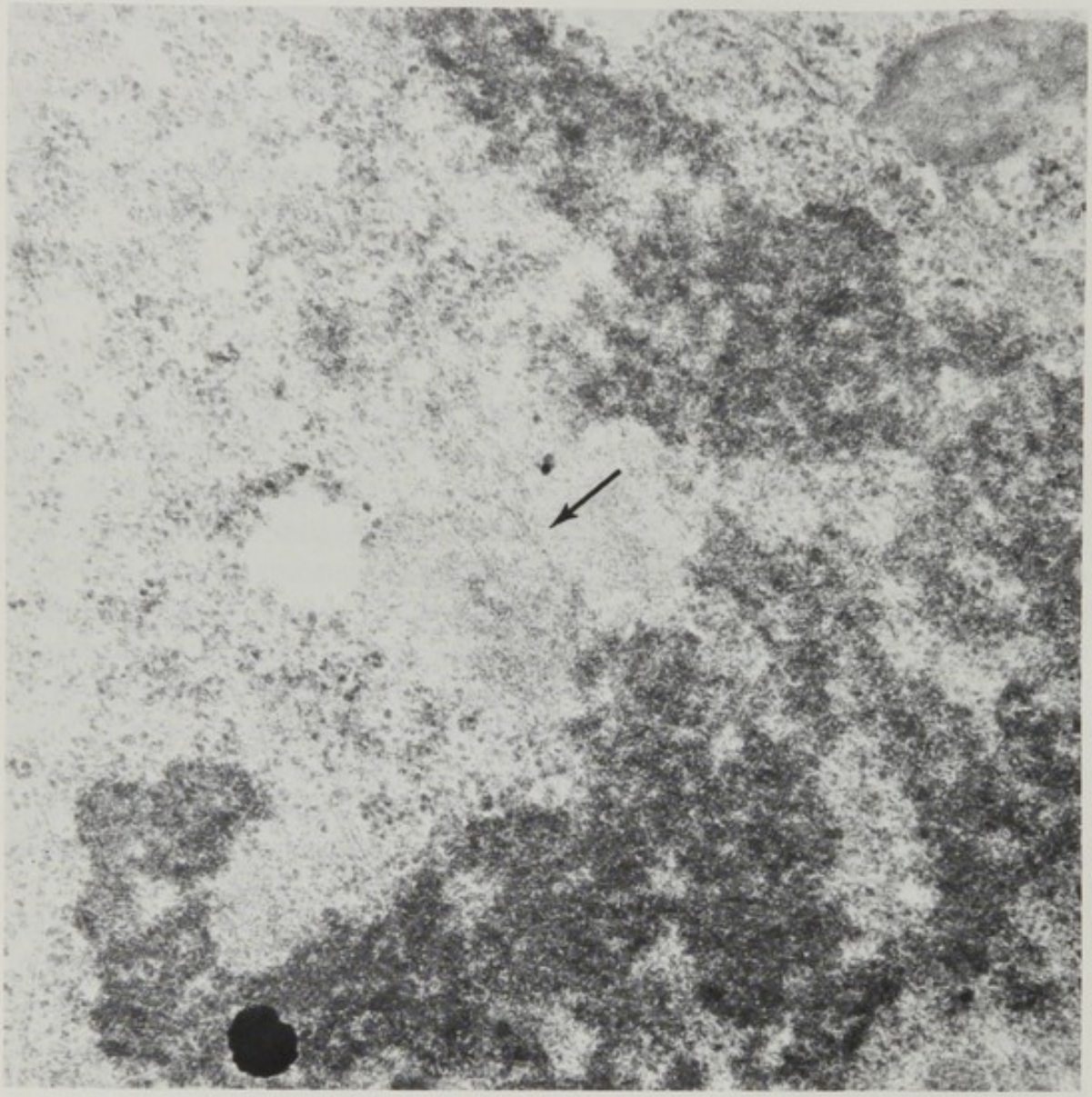

Fig. 10. An electron micrograph of S PCC of a HeLa cell. Spindle tubules (arrow) are attached to the pulverized chromatin at several points. (From Aula, 1970.)

chromosomes exhibited as many as six bands in PCC. Therefore, it appears that as chromosomes condense several bands seen in G_2 PCC merge to produce a single band in metaphase chromosomes (Unakul *et al.*, 1973).

Even though no mitotic apparatus was seen with PCC induction (Johnson and Rao, 1970), in a few instances (Aula, 1970; Sanbe *et al.*, 1970) microtubules have been found to be associated with PCC (Fig. 10). Ultrastructural studies of PCC by Matsui *et al.* (1972) also revealed the presence of a kinetochore in a prematurely condensed chromosome (Fig. 11). On the basis of these observations and those reported by Rao and Johnson (1972) on the fate of PCC in mitotic-interphase hybrids, it appears that the process of premature chromosome

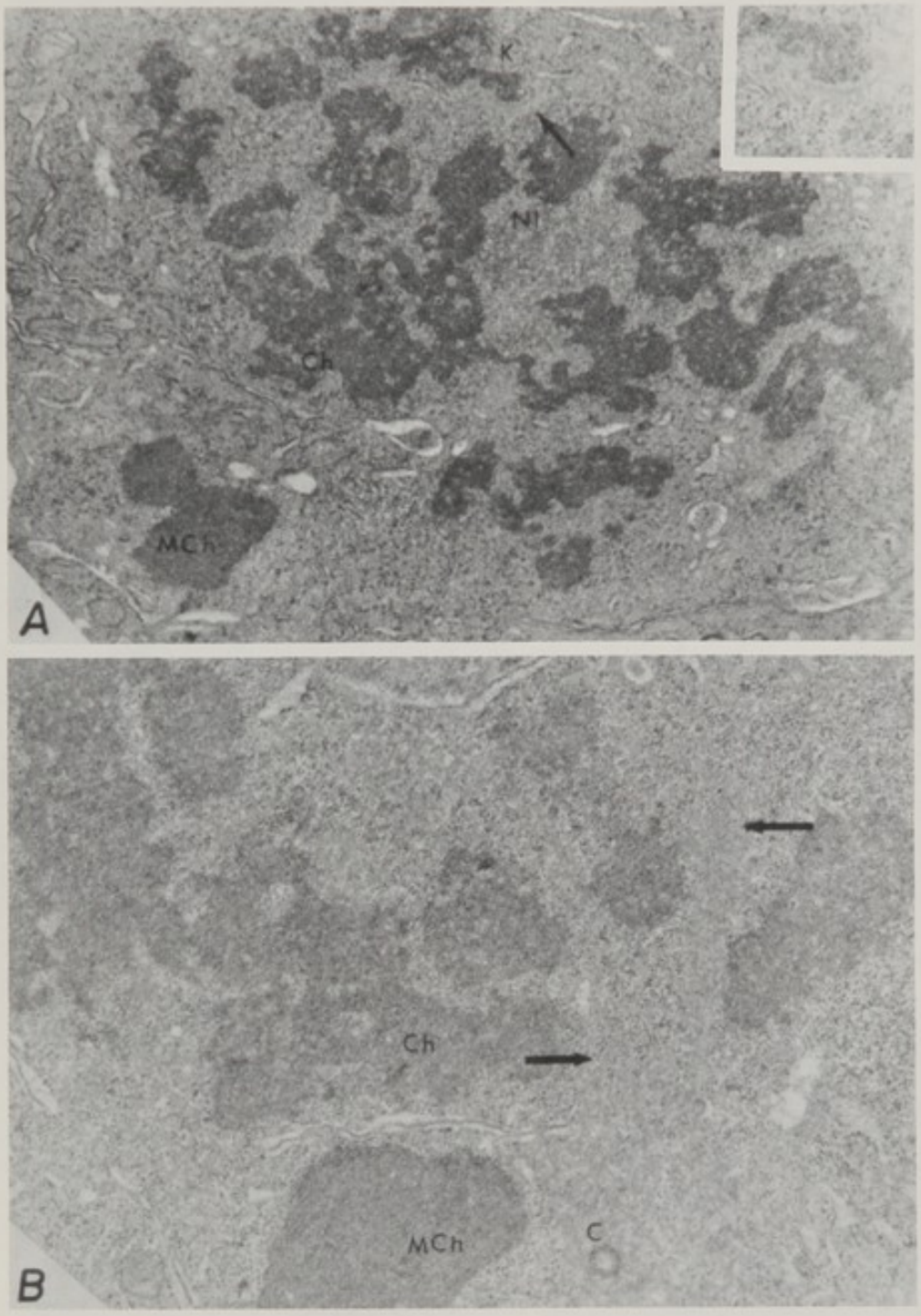

Fig. 11. (A) I-M cell at 20 minutes after cell fusion; condensed chromatin (Ch) and a granular nucleolus (Nl) originating from the interphase cell. A kinetochore (K) is attached to the condensed chromatin (arrow). A metaphase chromosome (MCh) is present in this field. ×8400. The insert shows a further enlargement (×16,800) of the kinetochore area. (B) Another I-M cell 20 minutes after cell fusion. Several spindle tubules (arrows) stretch to the condensed chromatin (Ch). The degree of condensation is different between interphase chromatin and metaphase chromosomes (MCh). Note the centriole (C). ×7200. (From Matsui *et al.*, 1972.)

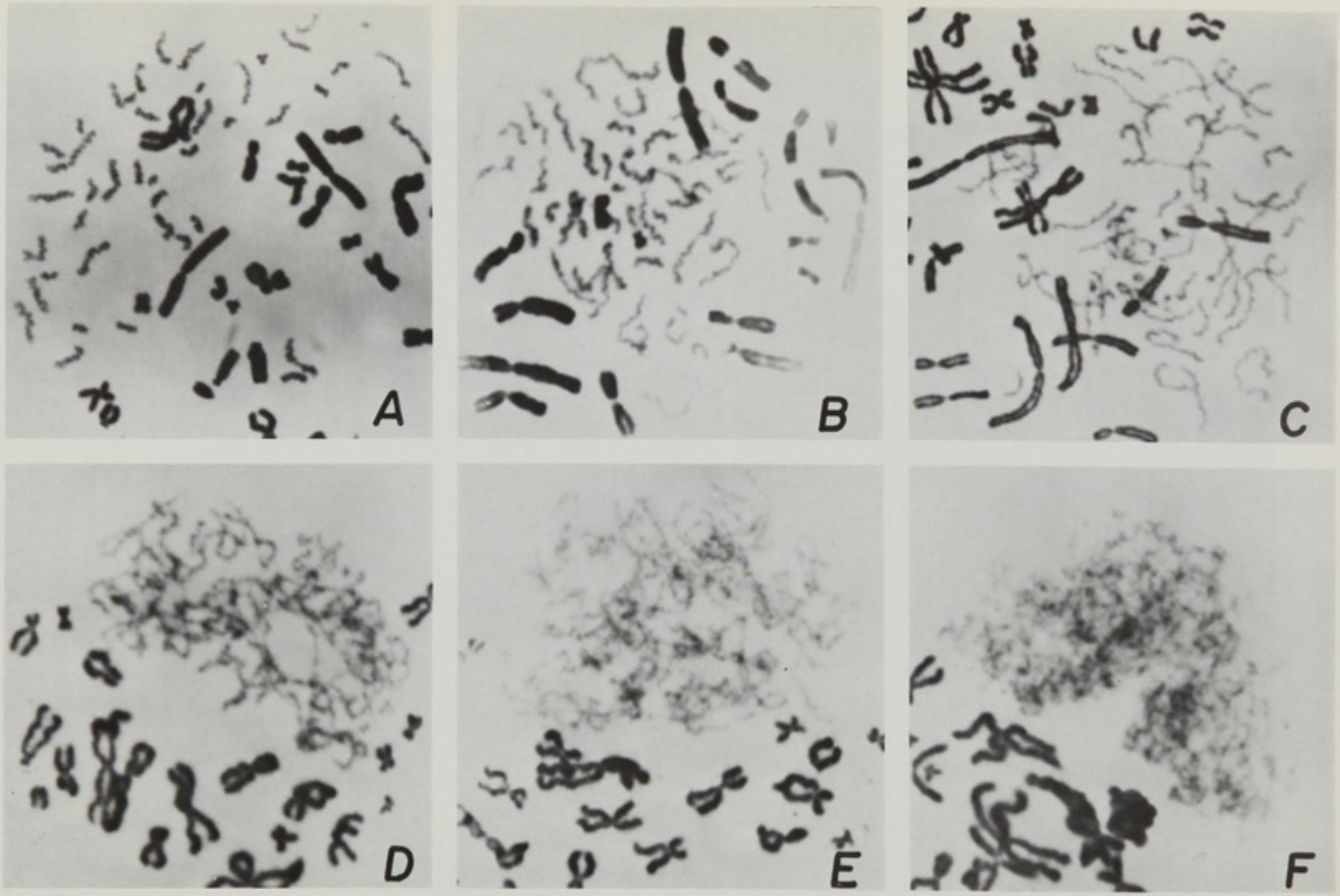

Fig. 12. PCC of human peripheral blood lymphocytes in G_1 phase showing different degrees of decondensation. The PCC were assigned decondensation values starting from the lowest value: (A) + 1; (B) + 2; (C) + 3; (D) + 4; (E) + 5 and (F) + 6. (From Hittelman and Rao, 1976a.)

condensation, like mitosis, is a natural and reversible phenomenon that eukaryotic cells go through under special conditions.

III. CHROMOSOMES OF DIFFERENTIATED CELLS

When dealing with differentiated cell systems, it is natural to ask during what phase of cell cycle are the cells switched from a proliferating to a differentiated state. Using spectrophotometric and flow microfluorometric methods it has been demonstrated that most of the differentiated cells possessed the G_1 (2C) amount of DNA. However, the discovery of the phenomenon of PCC made it possible to visualize the structure of the chromosomes of these nondividing cells. Using the mitotic HeLa cells as inducer cells, prematurely condensed chromosomes of chick erythrocytes, horse lymphocytes and bovine sperm were visualized (Johnson *et al.*, 1970). The PCC obtained from these three different systems are of G_1 type.

Recently, we have been reexamining the peripheral lymphocyte system during the initial stages of phytohemagglutinin (PHA) stimulation in the light of the phenomenon of premature chromosome condensation (Hittelman and Rao, 1976). It has been shown that the template activity of chromatin of lympho-

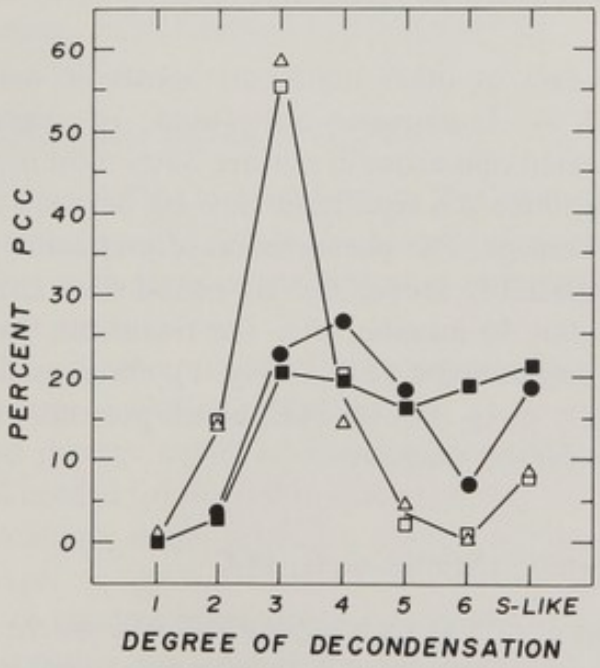

Fig. 13. Relative frequencies of PCC with various degrees of decondensation □——□, – PHA incubated at 37°C; △——△, ± PHA refrigerated overnight; ●——●, + PHA incubated 18 hours at 37°C; ■——■, + PHA incubated 22 hours at 37°C. (From Hittelman and Rao, 1976.)

cytes increased following stimulation with PHA (Hirschhorn *et al.*, 1969). We wanted to find out whether the conformational changes taking place in the chromatin during the initial stages of PHA stimulation, i.e., before the onset of DNA synthesis, would be reflected in the structure of PCC of these lymphocytes. Human peripheral lymphocytes were incubated with or without PHA for 18 or 24 hours and then separately fused with mitotic Chinese hamster ovary (CHO) cells. In all these treatments only G_1 type of PCC were observed, but they exhibited variable morphology, i.e., some were condensed and others were highly decondensed. Based on the degree of decondensation the PCC were grouped into six categories, the value of six being the most decondensed (Fig. 12). In the unstimulated control the PCC were predominantly of group 3, whereas group 4 was more frequent in cells after 18 hours of PHA stimulation (Fig. 13). Incubation for 24 hours shifted this frequency distribution curve even further to the right. These studies indicate that a stimulated population could be detected cytologically from an unstimulated one far in advance of the initiation of DNA synthesis. This method has proved to be valuable in evaluating the proliferative state of bone marrow of leukemic patients before, during, and after chemotherapy (Hittelman and Rao, in preparation).

IV. PREMATURELY CONDENSED CHROMOSOMES FOR STUDY OF CHROMOSOME ABERRATIONS

The effects of X-rays or other mutagenic agents on mammalian cells are commonly measured as chromosome aberrations, i.e., gaps, breaks and exchanges, when the treated cells arrive in mitosis. Since most of these agents cause mitotic delay, usually there is a significant time lag between the treatment and the visualization of damage. The phenomenon of premature chromosome condensation could considerably reduce this lag period since chromosome damage can be visualized within 30 minutes after the treatment. As evident from the morphology of the various types of PCC (Fig. 1), chromosome aberrations can be studied either in G_1 or G_2 PCC. S PCC, which presents a pulverized appearance, are not suitable for this purpose.

A. Chromosome Aberration Studies on G_1 PCC

Waldren and Johnson (1974) analyzed the PCC of a synchronized G_1 population of HeLa cells immediately after X- or ultraviolet-irradiation. X-Irradiation caused fragmentation of G_1 PCC in a dose-dependent manner. This relationship was linear up to doses of about 1800 rads with an average increase of 10–15 fragments for every 100 rads. The level of damage observed in G_1 PCC appeared to be considerably higher than that reported in mitotic chromosomes for a

comparable dose of X-rays. Even the finely chopped fragments of G_1 PCC are discrete and well condensed. A 2 hour incubation of cells after irradiation resulted in a rapid reduction in the number of fragments due to rejoining and repair. The rejoining of fragments is not associated with the uptake of ^{3}H-thymidine which indicates the absence of any significant amount of DNA synthetic activity. In contrast, the PCC of G_1 cells treated with UV light are not fragmented but they become elongated and attenuated resembling the morphology of S PCC. This structural alteration in G_1 PCC arising as a result of UV-irradiation is associated with unscheduled DNA synthesis. Recently Schor *et al.* (1975) have shown that the susceptibility of G_1 cells to UV-irradiation (with regard to the induction of S-like PCC) increases as the cells progress towards DNA synthesis.

These studies reveal that irradiation of G_1 cells with UV light induces changes in the structure of chromatin which on fusion with mitotic cells yield prematurely condensed chromosomes similar to those obtained from S phase cells. In other words, the G_1 cells which are going through unscheduled or repair synthesis following UV irradiation are functionally as well as structurally akin to those in S phase. X-Irradiation, on the other hand, has no effect on the conformational state of chromatin, since even the most finely chopped G_1 PCC exhibit a normal degree of condensation. Since no ^{3}H-TdR was incorporated into the chromosomes during the process of rejoining of X-ray-induced fragments it is believed that only a few bases are inserted to reunite the fragments (Waldren and Johnson, 1974; Painter and Young, 1972).

B. Chromosome Aberration Studies on G_2 PCC

Hittelman and Rao (1974a,b,c) used the G_2 PCC system extensively to study the nature of chromosome aberration formation in Chinese hamster ovary cells following treatment with physical and chemical mutagenic agents. The reasons for using this system are as follows:

1. The G_2 PCC are more extended than mitotic chromosomes and perhaps this would allow better resolution of damage.

2. The induced damage could be observed immediately rather than after a prolonged interval needed for the cell to reach mitosis.

3. The PCC technique might provide a direct method of measuring repair of chromosome damage.

4. The ability to regulate chromosome condensation by cell fusion could be helpful in understanding the relationship between chromosome condensation and aberration formation.

According to the procedure of Hittelman and Rao (1974a) CHO cells in exponential growth are X-irradiated (217.5 rad) and one half of the irradiated cells are immediately fused with mitotic CHO cells to induce PCC while the

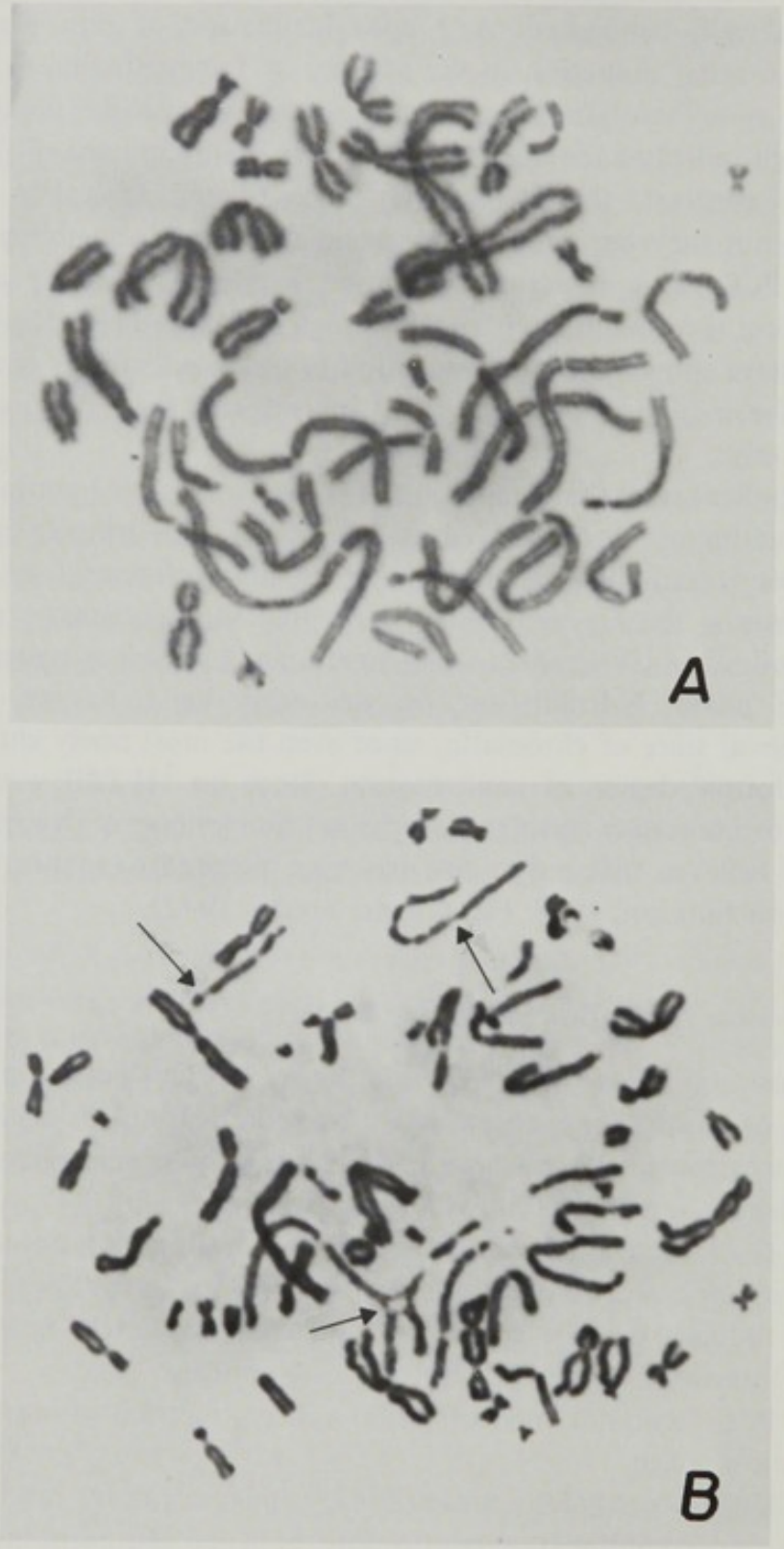

Fig. 14. Visualization of radiation damage in G_2 PCC of CHO cells. (A) G_2 PCC of an untreated cell; note that PCC are slender and less condensed. (B) G_2 PCC of a cell, treated with 217.5 rad of X-rays, exhibited gaps, breaks, and an exchange (arrows). (From Hittelman and Rao, 1974a.)

TABLE II
Comparative Estimates of Radiation Damage to CHO Cells by Scoring PCC and Mitotic Chromosomes[a]

Treatment	Type of chromosomes scored	Number of cells scored	Aberrations per cell		
			Gaps	Breaks	Exchanges
+ X-rays	PCC	50	2.86	2.50	0.50
− X-rays	PCC	50	0.28	0.06	0
+ X-rays	Mitotic	150	2.31	1.31	0.39
− X-rays	Mitotic	150	0.05	0.01	0.01

[a]From Hittelman and Rao (1974a).

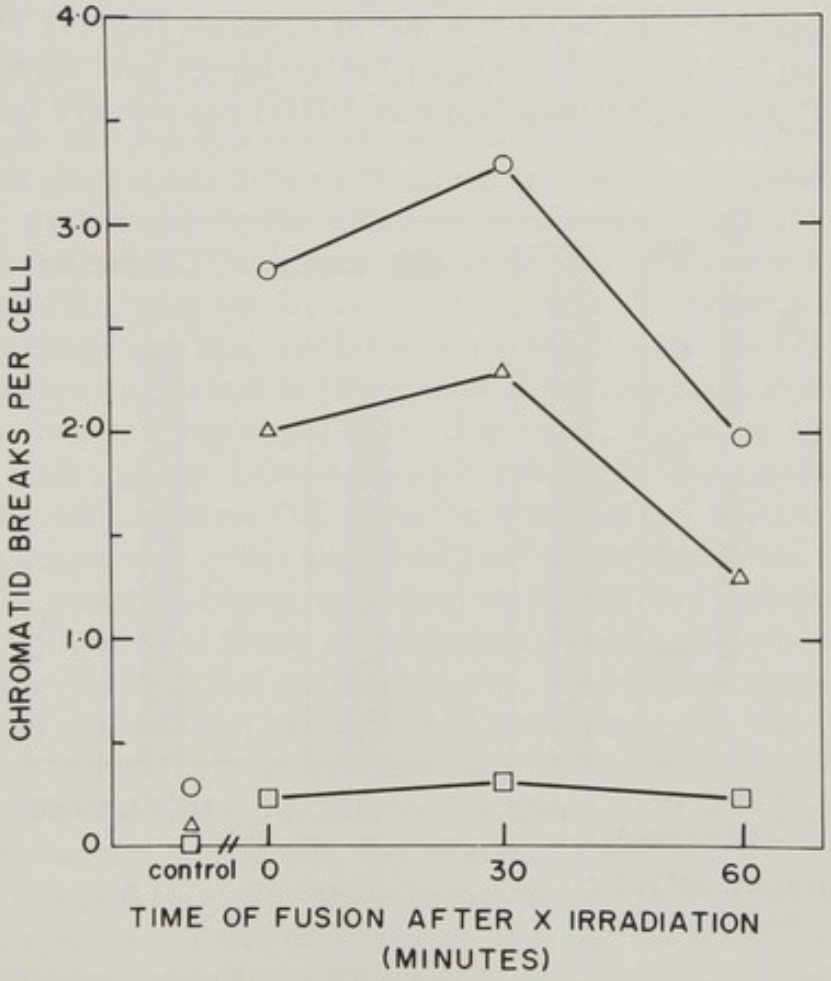

Fig. 15. The frequency of chromatid aberrations in G_2 PCC of CHO cells as a function of the time elapsed between X-irradiation and cell fusion. ○——○, gaps; △——△, breaks; □——□, exchanges. (From Hittelman and Rao, 1974a.)

remaining half is incubated with Colcemid (0.05 μg/ml) for 2 hours to obtain mitotic chromosome spreads. From Fig. 14 it is clear that chromosome aberrations could be scored on G_2 PCC. The extent of chromosome damage seen in G_2 PCC is compared with that in mitotic chromosomes (Table II). More chromosome damage was observed in the G_2 PCC than in mitotic chromosomes. These data indicate that scoring for chromosome damage in mitotic cells does not reveal the true extent of damage to the cells. One of the reasons for such a difference in the results of these two methods is probably the failure of heavily damaged cells to enter mitosis. In the older method, it is essential for the cell to enter mitotsis in order to be scored for damage. According to the PCC method, even those cells that are blocked in G_2 could be scored for chromosomal aberrations. The second factor is the time available for repair between the treatment and the visualization of chromosome damage. By using the PCC method it is possible to estimate the repair as a function of time after irradiation. Estimation of chromosome damage in G_2 PCC induced at 30 minutes and 60 minutes after X-irradiation revealed a significant decrease in the frequency of chromatid gaps and breaks but not in that of exchanges (Fig. 15). This suggests that gaps and breaks can be repaired but exchanges once formed are not repairable. This study (Hittelman and Rao, 1974a) also provided some indirect

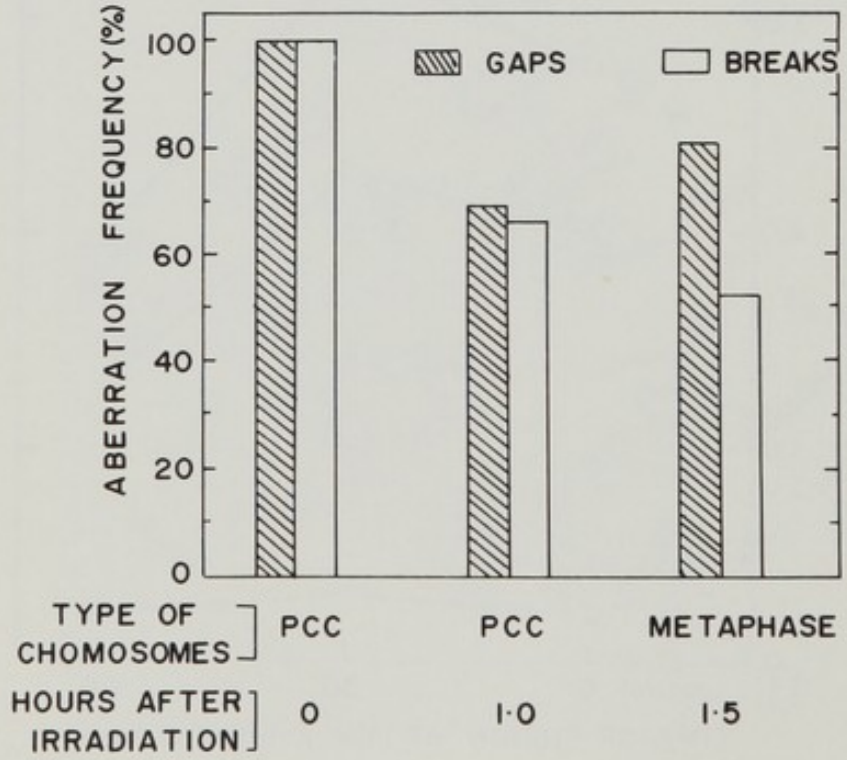

Fig. 16. The frequencies of gaps and breaks in PCC of CHO cells as compared to those in metaphase chromosomes. The number of aberrations (gaps and breaks) scored in PCC at 1 hour after irradiation and metaphase chromosomes were expressed as a percent of that observed in PCC at 0 hours after irradiation. (From Hittelman and Rao, 1974a.)

evidence about the nature of chromatid gaps. The kinetics of repair for gaps and breaks in G_2 PCC follows a similar pattern as can be seen in Fig. 16 (left and middle pair of columns). On this basis one would expect the ratio between gaps and breaks in mitotic cells to be similar to that observed in G_2 PCC. However, relatively more gaps were observed in metaphase chromosomes than expected (Fig. 16). This may be due to one of the following reasons. (1) Gaps seen in metaphase chromosomes may represent chromatid breaks which are hidden in the condensed chromosome as suggested by Comings (1974). (2) There are two types of gaps, one of which could be seen in both mitotic chromosomes and G_2 PCC, while the other type could be traced only in the mitotic chromosomes which are more condensed than the G_2 PCC. The studies with UV-irradiation and alkylating agents support this possibility (Hittelman and Rao, 1974b).

C. Nature of Chromosome Gaps

Alkylating agents and UV-irradiation are known to cause structural alterations in DNA (Lawley, 1966; Setlow, 1968; Smith and Hanawalt, 1969) as well as in chromosomes (Chu, 1965; Faberge, 1951; Humphrey *et al.*, 1963; Kirby-Smith and Craig, 1957; Revell, 1959; Swanson, 1944) but the relationship between these two events is not yet completely understood. When G_2 cells are treated with these agents little visible damage (no breaks or exchanges) is seen except for the increase in the frequency of chromatid gaps during the first mitosis after treatment. The nature of these gaps has been probed by Hittelman and Rao (1974b) using the G_2 PCC system. As in the previous studies with X-rays (Hittelman and Rao, 1974a) an exponential culture of CHO cells were exposed to nitrogen mustard (a bifunctional alkylating agent), trenimon (2,3,5-triethylene iminobenzoquinone-1,4; a trifunctional alkylating agent) or UV irradiation (100 ergs/mm^2). One half of the treated cells were fused immediately with mitotic cells to induce PCC while the remaining half was incubated for 2 hours with Colcemid to collect mitotic cells and prepare metaphase chromosome spreads. The extent of damage was scored on G_2 PCC and metaphase chromosomes. The frequency of breaks and exchanges per cell in the treatments was no different from the untreated control. However, the incidence of gaps in mitotic chromosomes as a result of treatment with alkylating agents was three to four times greater than that in G_2 PCC (Table III). In the case of UV-irradiation, G_2 PCC exhibited more gaps than mitotic chromosomes. These opposing trends indicate that we are dealing with two structurally different types of gaps. The ability to distinguish these gaps lies in the fact that the degree of condensation in G_2 PCC is a different order of magnitude than that of mitotic chromosomes. The G_2 PCC are long and extended because they are less condensed than the mitotic chromosomes. This difference in the degree of condensation seems to bring out the molecular differences underlying the gap formation. Thus agents,

TABLE III
Effect of UV-Irradiation and Alkylating Agents on the Incidence of Gaps in the Chromosomes of CHO Cells[a]

Type of chromosomes scored	Treatment	Number of cells scored	Number of gaps	Gaps per cell	Net increase of gaps per cell due to treatment	Ratio of gaps (mitotic:PCC)
PCC	+HN2	50	37	0.74	0.52	3.38
PCC	–HN2	50	11	0.22		
Mitotic	+HN2	100	186	1.86	1.76	
Mitotic	–HN2	100	10	0.10		
PCC	+Trenimon	50	30	0.60	0.40	4.55
PCC	–Trenimon	50	10	0.20		
Mitotic	+Trenimon	100	186	1.86	1.82	
Mitotic	–Trenimon	100	4	0.04		
PCC	+UV	50	19	0.38	0.22	0.23
PCC	–UV	50	8	0.16		
Mitotic	+UV	100	7	0.07	0.05	
Mitotic	–UV	100	2	0.02		

[a]From Hittelman and Rao (1974b).

such as BUdR (Zakharov and Egolina, 1972) and actinomycin D (Hsu *et al.*, 1973) which interfere with the condensation of chromosomes by causing despiralization of chromatin, may produce gaps which become visible in the highly condensed mitotic chromosomes but not in the less condensed G_2 PCC. The chromatid gaps seen in the first mitosis after treatment of G_2 cells with alkylating agents may be of this type. The second type of gaps are those in which there is a discontinuity in one or both the strands of the DNA double helix. This type of gaps are commonly seen after X- or UV-irradiation. The incidence of these gaps is higher in G_2 PCC than in mitotic chromosomes (Table II). It has also been shown that some of these gaps can be repaired following treatment and hence the high frequency of these aberrations seen in G_2 decrease by the time the cell arrives in mitosis. These experiments suggest that the gaps seen in mitotic chromosomes are of two types: one due to an alteration in the first order of chromosome condensation, probably due to single-stranded regions in DNA, and the second due to an alteration in the second stage of condensation, probably a result of an altered DNA-protein binding (Hittelman and Rao, 1974b).

V. CONCLUSIONS

The phenomenon of premature chromosome condensation has proved useful because:

1. It has made it possible to visualize the chromosomes of not only interphase cells but also of differentiated and nondividing cells.

2. It has also demonstrated that interphase chromatin can be condensed into discrete structures before or after DNA replication but not at the exact site of replication. Consequently it has helped to pinpoint the sites of replication on a chromosome at a given time.

3. It has provided a visual evidence for the existence of a chromosome condensation cycle which is tightly coupled to the cell division cycle.

4. It has proved to be superior over the classic method of scoring mitotic chromosomes in evaluating damage due to mutagenic and clastogenic agents. This method is uniquely suited in evaluating chromosome damage and repair after treatments which usually block the cells in G_2 temporarily or permanently.

5. By the application of this technique it is possible to distinguish two types of chromatid gaps, one due to an alteration in the first order of chromosome condensation, probably due to single-stranded regions in DNA and the second due to an alteration in the second stage of condensation, probably a result of an altered DNA protein binding.

ACKNOWLEDGMENTS

This investigation was supported by grants CA-16480, CA-11520, and CA-14528 from the National Cancer Institute, DHEW, GM-23252 from the Institute of General Medical Sciences, DHEW, and VC-163 from the American Cancer Society.

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7

New Chromosome Techniques

BERNARD DUTRILLAUX

I. INTRODUCTION

For many years, few major advances have been made in the field of cell culture, which constitutes an essential starting point for cytogenetic investigations. The widely used techniques derived from the method which Moorhead *et al.* described in 1960 for blood cell cultures permit convenient analysis of human karyotypes. Nonetheless, for certain cases, one must still rely on fibroblast cultures or amniotic cells, or even direct examination of marrow or germinative cells.

Apparently satisfied with such methods, cytogeneticists have not directed considerable attention toward developing new methods for obtaining cells that are undergoing division. Until the past few years, a similar situation existed for the staining and observation of chromosomes, but Caspersson and his associates stimulated a sudden change when their findings were published in 1968. In effect, they demonstrated with the fluorescent microscope that chromosomes are not homogeneous organelles. They possess very precise coloration qualities, which permit identification of numerous structures or bands that are characteristic of each chromosome pair.

Two years passed before the first applications with human chromosomes were undertaken (Caspersson *et al.*, 1970). Such a delay was surprising, when one considers the extraordinary activity which occurred in cytogenetics thereafter. In fact, during that period, other published material concentrated on the metaphase and interphase nuclei, furnishing evidence for a relationship between structural heterochromatin and satellite DNA (Yasmineh and Yunis, 1969; Arrighi *et al.*, 1970; Jones, 1970; Pardue and Gall, 1970; Yunis *et al.*, 1971). The technique developed by Yunis *et al.* (1971), in particular, set the groundwork for another set of banding techniques based upon thermal treatment, Giemsa staining, and use of the light microscope.

First, there was the R-band method (Dutrillaux and Lejeune, 1971), which was promptly followed by various G-band techniques (Sumner *et al.*, 1971; Drets and Shaw, 1971; Schnedl, 1971). Also, in 1971, a second type of G-band technique, using enzyme digestion, was reported (Dutrillaux *et al.*, 1971; Seabright, 1971; Finaz and de Grouchy, 1971).

Another set of techniques, using BUdR (5-bromodeoxyuridine), which is comparable to thymidine, was also reported by Palmer (1970) and by Zakharov *et al.* (1971). These techniques, which were improved by use of fluorochromatic substances such as acridine orange (Dutrillaux *et al.*, 1973) and Hoechst derivative 33258 (Latt, 1973), became the most popular, since they permitted one to obtain a considerable variety of bands which, as we shall see in Section III,A, depend on chromosome replication.

Finally, certain bandings characteristic of heterochromatin, including G_{11}-bands (Bobrow *et al.*, 1972; Gagne and Laberge, 1972) or to telomeric regions, such as the T-bands (Dutrillaux, 1973), also deserve mention.

Most of these methods consist of treating or staining stationary chromosome preparations and are described first. Methods which utilize BUdR, on the other hand, consist of treating living cells during DNA replication. These are dynamic methods and are described subsequently, along with other methods which are of less interest. After a brief overview of findings obtained for other types of cells, we have, as a conclusion, attempted to analyze various hypotheses concerning the origin of bands.

II. METHODS FOR BANDING FIXED CHROMOSOMES

A. Staining with Quinacrine Mustard: Q-Bands

Staining with quinacrine mustard has led to many advances in human cytogenetics. Each chromosome pair is clearly depicted with this stain (Caspersson *et al.*, 1970). Unfortunately, although it can be produced by simple methods, it requires the use of ultraviolet light for microscope observation. Moreover, its fluorescent qualities are very low, so that there is considerable difficulty in taking photographs. Hence, this method has been replaced by Giemsa staining methods for systematic studies. Nonetheless, quinacrine mustard is the only substance which possesses high specificity for certain chromosome segments.

The Y chromsome, in particular, exhibits an intense fluorescence at the end of its long arm (Fig. 1). This permits not only easy recognition, but clear exposure of its polymorphism from one individual to another (Bobrow *et al.*, 1971). This characteristic, which was identified by Zech in 1969, was widely used for furnishing evidence of the Y chromosome in interphase nuclei of a broad variety of cells (Pearson, 1970), ranging from fibroblasts to spermatozoa. Thus, after a simple smear of epithelial cells from the mucous membrane of the mouth, the Y chromosome can be identified. This use of the Y body complements use of the Barr body for simple determination of the sex chromosomes. When staining is performed simultaneously with quinacrine mustard and quinacrine orange, one can even distinguish, in cases of sexual aneuploidism, the nucleoli and the interphase X and Y bodies, enabling the study of the spatial relationships among them (Fig. 2).

Other regions, such as the centromeric regions of chromosomes 3, 4, and 13 can also be specifically stained with quinacrine mustard. In addition, the satellites of certain acrocentric chromosomes can be stained. Uptake of the stain varies considerably from one chromosome to its homologue and from one individual to another. When one of these segments is highly fluorescent, it is visible in the interphase nuclei and can be confused with the Y chromosome.

B. C-Bands and Heterochromatin Staining

Structures considered within the category of C-bands are not chromosome bands in reality. Instead, they constitute a particular type of chromatin, constitutive heterochromatin, whose location is essentially opposite the centromere.

Various staining techniques have been reported (Arrighi and Hsu, 1971; Yunis *et al.*, 1971; Dutrillaux and Couturier, 1972; Sumner, 1972) in which the preparations can be treated with, for example, saline solution, alkaline solution, urea, barium hydroxide often with heat.

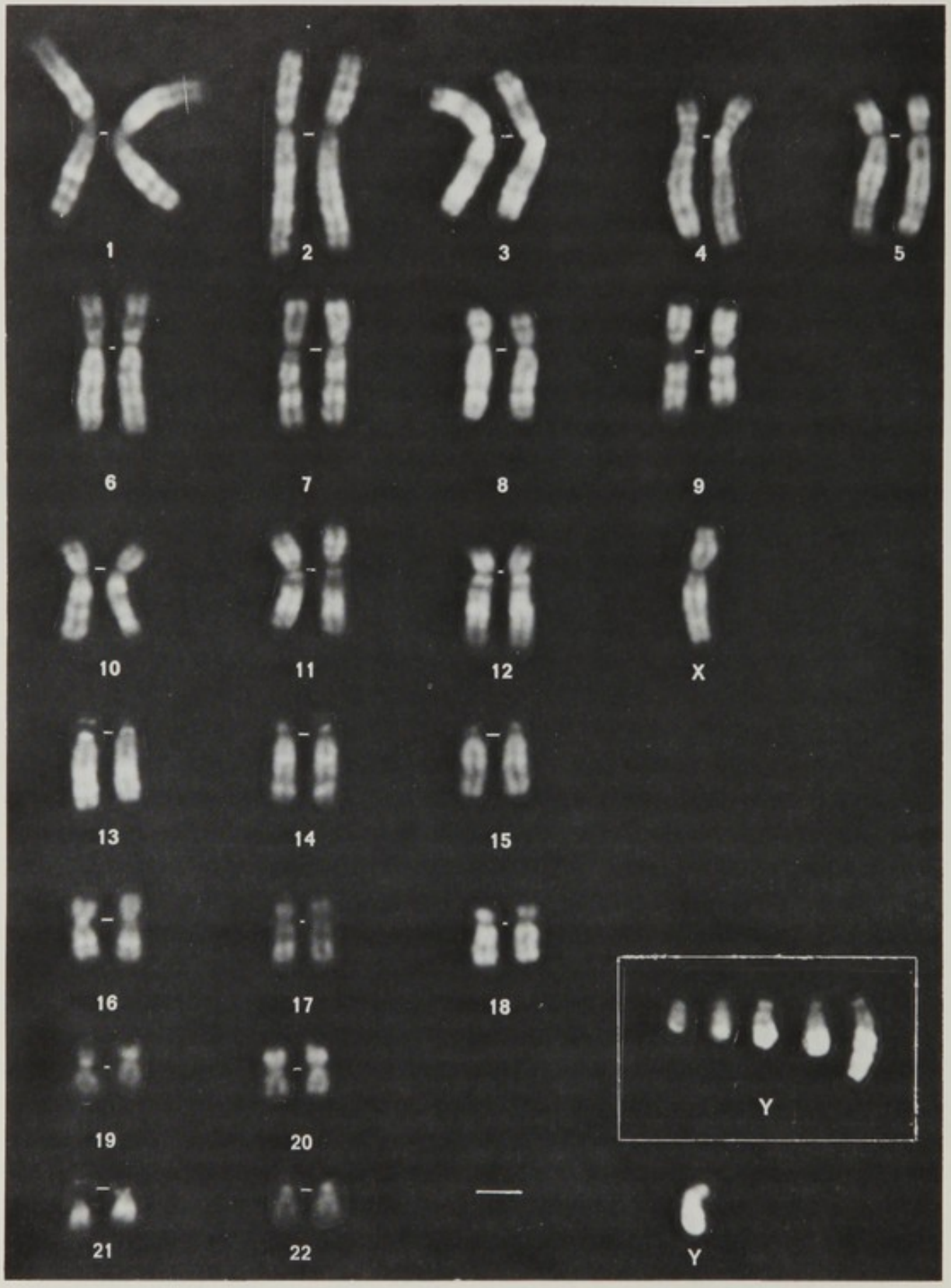


Fig. 1. Normal male karyotype with Q-bands. The varying length of the Y chromosome in normal individuals is illustrated.

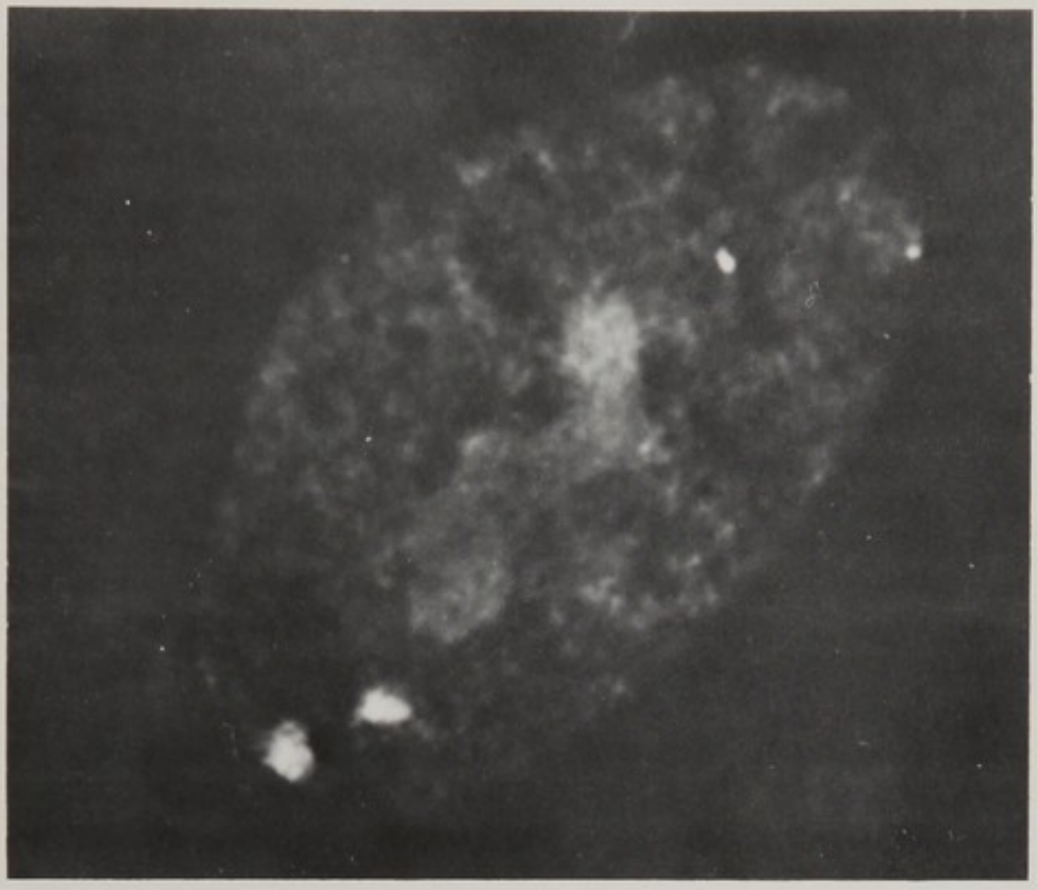


Fig. 2. Interphase nucleus (tetraploid) of a subject with a 47, XXY sex complement. The two most highly fluorescent masses are the X bodies, and the two points are the Y bodies. The nucleolus is visible at the center of the nucleus.

After Giemsa staining, the chromosomes acquire limited coloration. The chromatids are very pale, while the pericentromeric regions and the long arm of the Y chromosome are relatively intensely stained. The chromatids, which are sometimes homogeneous, often carry traces of G-bands.

Practical applications of these banding methods are relatively limited. Another type of technique, which consists of staining the preparations with a Giemsa solution having an alkaline pH–G_{11} (Bobrow *et al.*, 1972; Gagne and Laberge, 1972)–permits one to stain only a few of the C-bands, and is regarded as more selective. Hence, this technique can be successfully used for analysis of certain precise alterations of chromosomes.

C. R-Bands and T-Bands

In comparison with the many varied techniques for C- and G-bands, methods for observing R- and T-bands are not very numerous. R-bands (Dutrillaux and

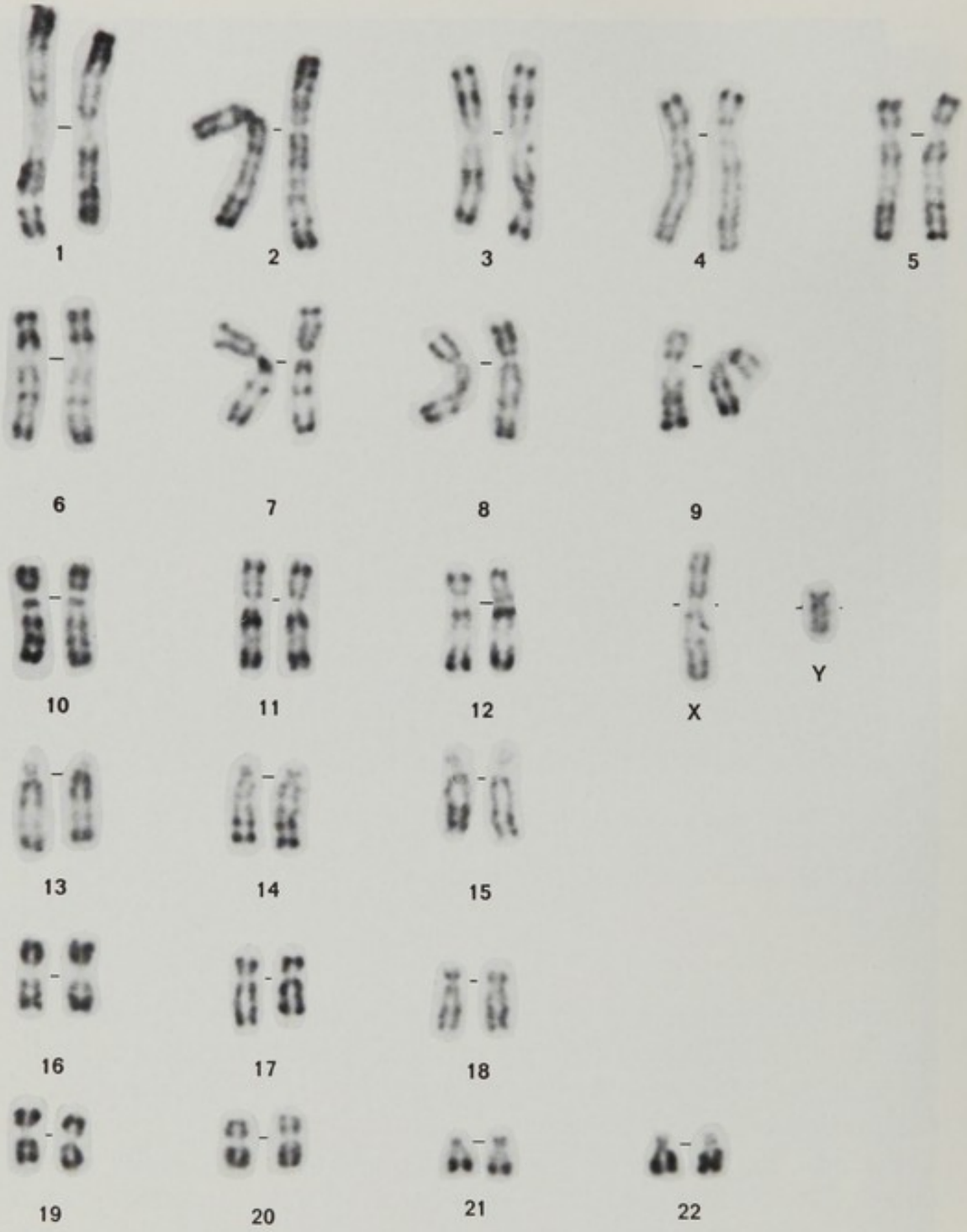


Fig. 3. Normal male karyotype, R-bands.

Lejeune, 1971) have staining characteristics opposite of those for Q- or G-bands (Paris Conference, 1971; Dutrillaux *et al.*, 1972). One of their characteristics is that they are almost always positive at the ends of the chromatids. This permits precise analysis of the morphology of chromosomes (length and centromere index) and the convenient observation of minor structural alterations which frequently affect the telomeres (Lejeune *et al.*, 1973).

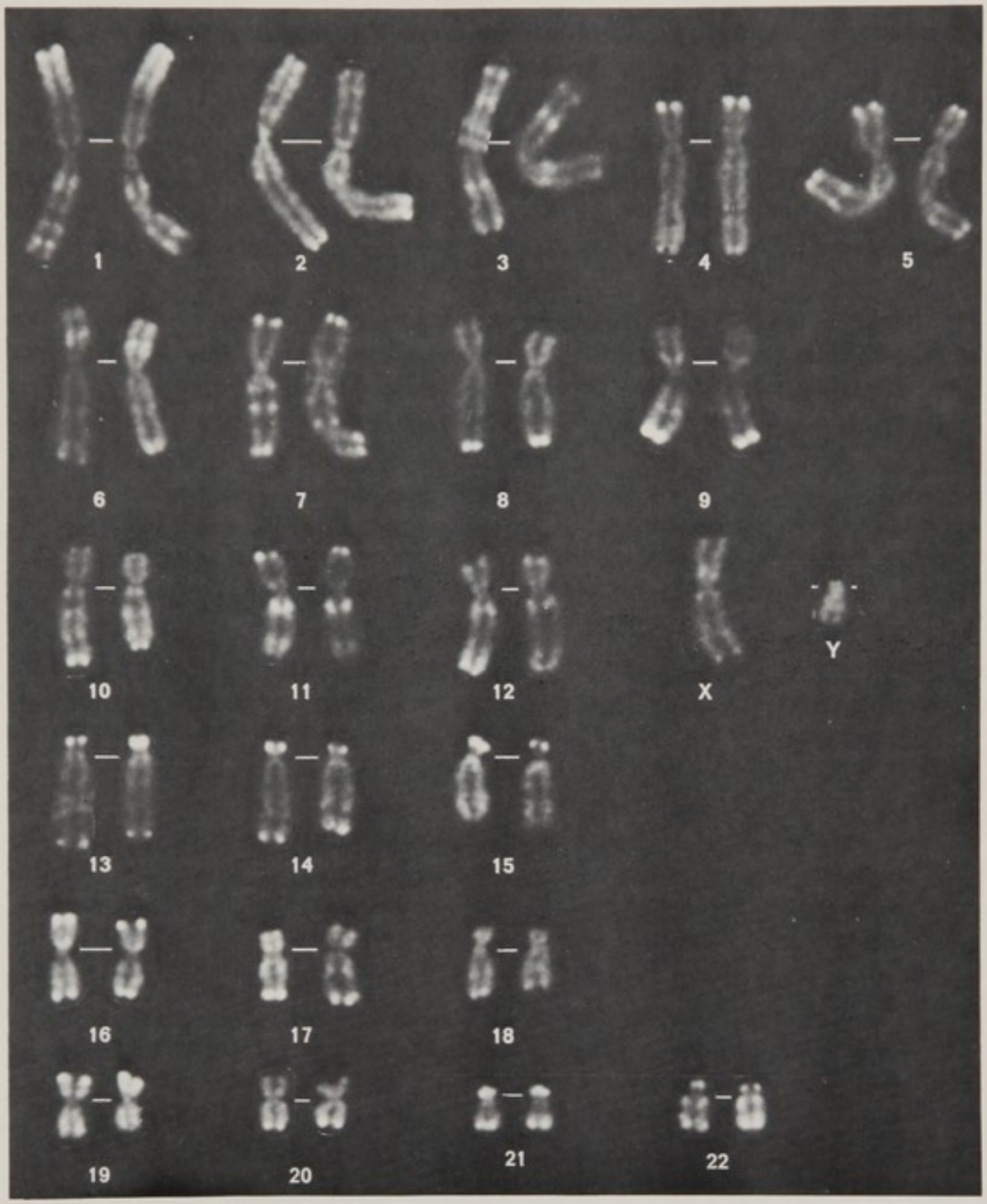


Fig. 4. Normal male karyotype, T-bands. The end of chromatids are preferentially stained.

T-bands represent a fraction of the R-bands (Dutrillaux, 1973)—the fraction which is precisely located, with only rare exceptions, at the terminal portion of the chromatids. They also represent the segment of R-bands that is most resistant to heat treatment. In fact, it is possible, by means of gradual treatment, to obtain several stages between R-bands and T-bands.

These two patterns can be observed either after Giemsa staining (Fig. 3) or after use of acridine orange (Fig. 4). However, for practical reasons, it is preferable to use the former method of staining for R-bands and, in order to obtain a better contrast, to use the latter method for T-bands.

D. G-Bands

Possibilities for obtaining G-bands appear to be innumerable. Indeed any staining of the Giemsa type makes them visible to such an extent that a re-examination of old chromosome records often permits observation of what we would now call G-bands. Two types of techniques appear to be suitable. One consists of thermal treatment within a concentrated saline solution [methods derived from Yunis *et al.* (1971) and Sumner *et al.* (1971)]; the other type consists of digestion by proteolytic enzymes (methods derived from that developed by Dutrillaux *et al.*, 1971). The latter, in particular, after modification by Seabright (1971), continues to be widely used because of its great ease. However, it has the disadvantage of not being highly reproducible.

Localization of G-bands is the same as for Q-bands, although there are some distinctions, such as the G-positive staining of some regions near the centromere (Fig. 5).

E. Use of Several Techniques with the Same Preparations

For analysis of difficult cases, such as distinct or complex chromosome translocations, or in species in which there may be great resemblance among several chromosomes, it may be necessary to analyze the same metaphase using several different techniques. The order in which the steps are performed is not haphazard, and, since few descriptions have been provided, the following points are given:

1. Staining by the Giemsa method, which permits observation of chromosomes in their classic form, is always possible prior to implementing other staining techniques, with the exception of enzyme digestion. It even appears that the Giemsa stain permits better conservation of slides provided that they are not mounted. The immersion oil can be removed with toluene.

2. Staining with quinacrine mustard, and probably with all other fluorochromes, and observation under ultraviolet light does not prevent subsequent use of other techniques. The fluorochrome which has been employed can be removed by water and alcohol rinsing.

3. G-band methods (except for enzyme digestion) can be used thereafter.

4. The R-band method can be easily employed for preparations that are stained for G-bands (except with enzyme digestion). It is very difficult but not

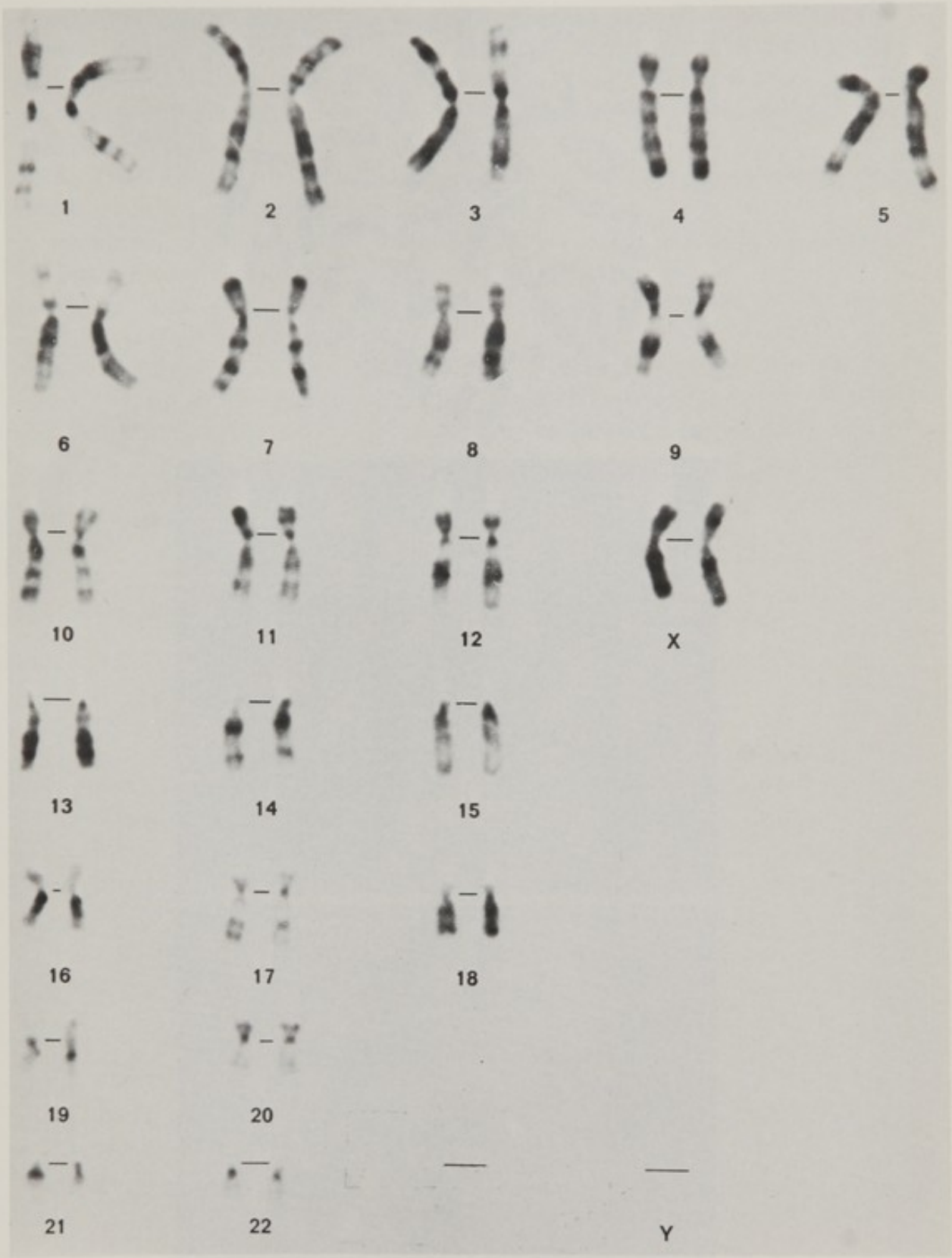
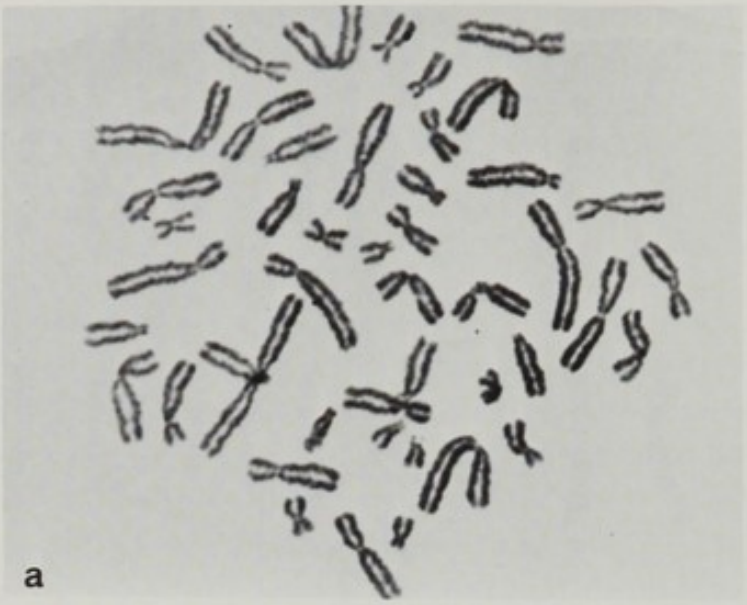


Fig. 5. G-banded karyotype.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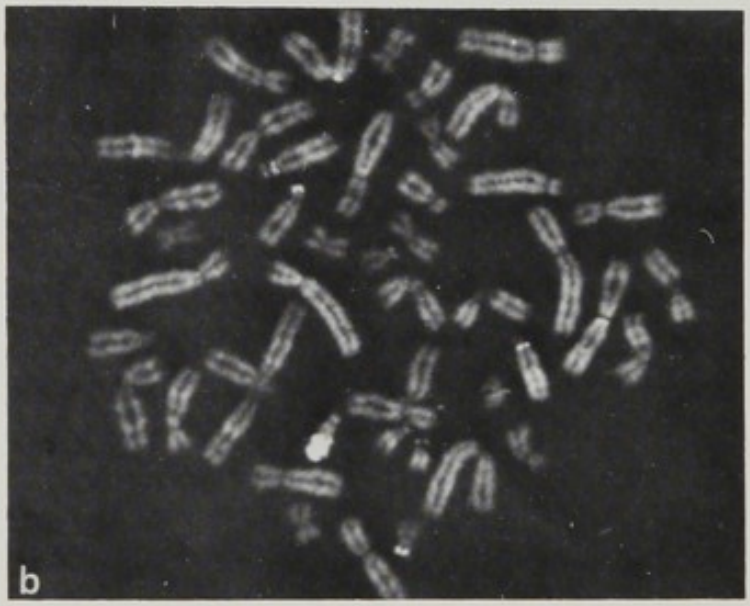
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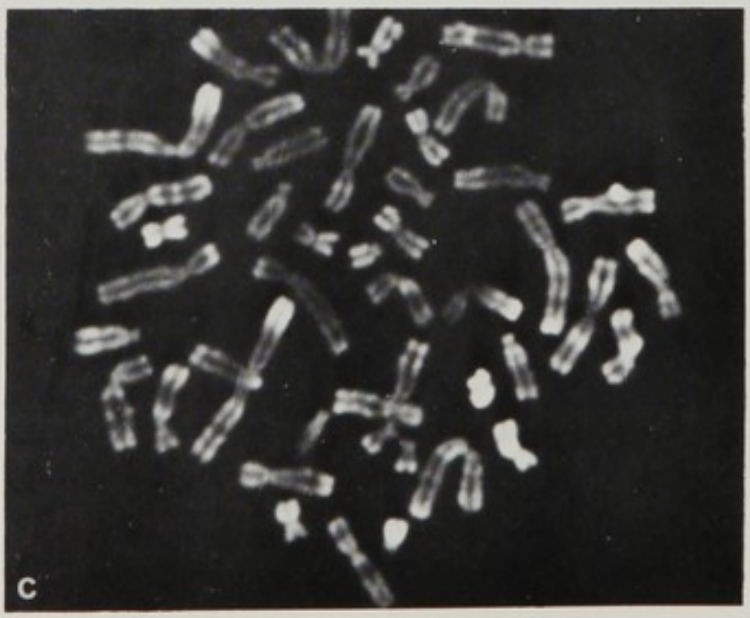
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impossible (Dutrillaux, 1975b) to obtain G-bands after R-bands. When the R-band treatment has been done, one can stain either by the Giemsa method (Dutrillaux and Lejeune, 1971) or with acridine orange (Bobrow *et al.*, 1972). Quinacrine mustard, on the other hand, furnishes very poor results, although it is still possible to identify the Y chromosome.

5. The T-band method can be utilized after each of the methods enumerated above, and the results are very consistent.

6. Finally, a C-band method can be used. It is possible to observe coexistence of T- and C-bands (Dutrillaux and Covic, 1974; Scheres, 1974).

Hence, it is theoretically possible, with a single preparation, to observe a classic staining, then the Q-bands, and then the G-, R-, T-, and C-bands for the same chromosomes. However, the various procedures tend to decrease the quality of the images, and, in reality, it is difficult to proceed beyond three or four different techniques. In practice, we have obtained the best results by using conventional staining, followed by quinacrine mustard (Q-bands), and then by heat treatment and staining with acridine orange (R- and T-bands). In this way, it is possible to observe complementary features (Fig. 6).

F. Conclusion

There are innumerable banding methods; we have described only those which, in our opinion, appear to be the most typical, furnish the most consistent results, and have served as models for other procedures.

Widespread use of these techniques in all laboratories of reasonable size developed very rapidly. However, very few laboratories use them systematically for all types of chromosome study. To our knowledge, only the technique for G-bands (enzyme digestion) and R-bands are routinely used in that manner.

This group of banding methods has now been available for several years, and, upon reflection, it is clear that there will be further expansion of the number of structures which can be studied without additional procedures, particularly without preliminary treatment of living cells. The limiting factors are the reduced length of chromosomes, even prophasic chromosomes, and the resolving power of light microscopes. Hence, the number of listed structures has increased from 322 (Paris Conference, 1971) to nearly 450 (Prieur *et al.*, 1973; Schnedl, 1973), then to 606 (Skovby, 1975), and finally has surpassed one thousand (Yunis and Sanchez, 1975; Yunis, 1976).

It should also be noted that cells which permit observation of more than 300 to 400 bands per haploid genome are only rarely observed, except for the synchronization method recently described by Yunis (1976).

Fig. 6. Cell from a patient having a 5p– chromosome defect. (a) Staining with Giemsa (G-bands); (b) quinacrine mustard (Q-bands); and (c) acridine orange (R-bands).

III. DYNAMIC METHODS

Under this heading, we shall consider methods which, by treatment of living cells and, ultimately, by subsequent procedures, permit identification of chromosome structures.

Autoradiography of chromosomes, following incorporation of tritiated thymidine (Taylor *et al.*, 1957; Taylor, 1960), was the first of these techniques to be developed. However, its long-term use in human cytogenetics in particular (German, 1962) does not permit one to regard this as a new technique. Moreover, it has never given results as accurate as those obtained from banding methods. In practice, this method has been widely replaced by those which rely on BUdR, which was first used in cytogenetics about the same time (Hsu and Somers, 1961), although 10 years passed before notable results were obtained with it (Palmer, 1970; Zakharov *et al.*, 1971). Other procedures, for example, introduction of actinomycin D, have also been recently developed, but, at the present time, their appeal seems much more limited, and we shall only describe them briefly.

A. BUdR Treatment

Kaback *et al.* (1964), who were the first to use BUdR in human genetics, regarded this product as capable of accentuating secondary constrictions of chromosomes 1, 9, and 16. Shortly thereafter, Palmer and Funderburk (1965) demonstrated that certain chromatid segments could be lengthened. In 1968, Zakharov and Egolina obtained excellent images with hamster cells. In 1970, Palmer discovered that certain human chromosomes could be identified, particularly the late replicating X chromosome. This was promptly confirmed by Zakharov *et al.* (1971) and Baranovskaya *et al.* (1972). Finally, use of fluorochromes (Dutrillaux *et al.*, 1973a; Latt, 1973) greatly facilitated possibilities of BUdR-induced banding, and techniques expanded after that point.

Presently, it is possible to obtain all band types by means of BUdR treatment, and, moreover, the existence of evidence concerning chromatid asymmetry permits analysis of chromatid exchanges (Zakharov and Egolina, 1972; Latt, 1973; Dutrillaux *et al.*, 1974; Perry and Wolff, 1974).

In order to obtain the various chromatid modifications, BUdR is used in varying proportions at different times, in a continuous or discontinuous manner.

In strictly descriptive terms, BUdR produces several chromatid modifications which can be summarized as follows: symmetrical elongation (Palmer and Funderburk, 1965); asymmetrical elongation (Zakharov and Egolina, 1972); decreased intensity under Giemsa staining or under staining with Hoechst product 33258 (Zakharov and Egolina, 1972; Latt, 1973); modification of staining by acridine orange: red and green staining (Dutrillaux *et al.*, 1973a,

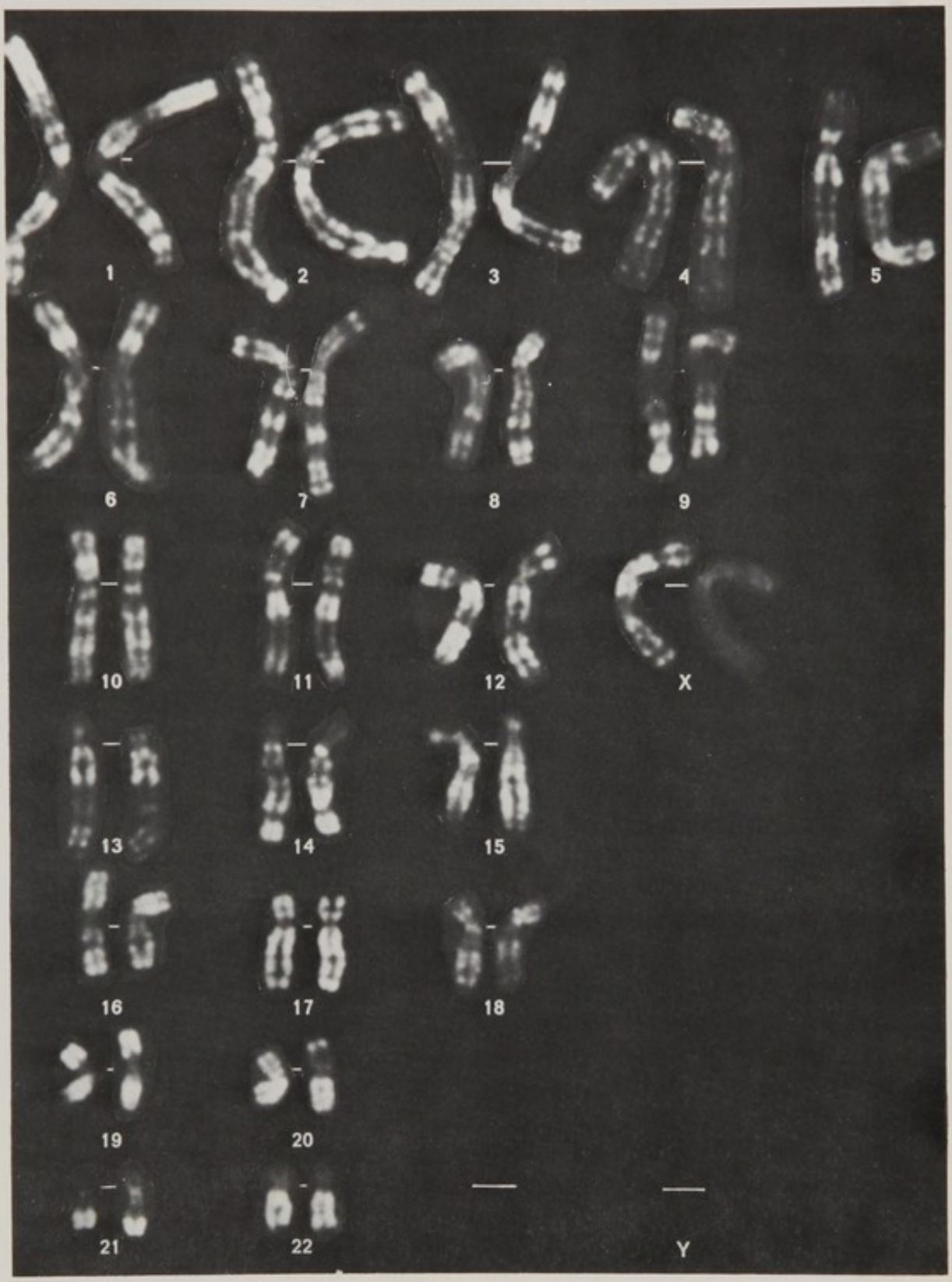

Fig. 7. R-bands observed after BUdR treatment and staining with acridine orange. The late-replicating X chromosome is lightly stained.

1974); and modification of response to heat treatments at 87°C and 65°C (Dutrillaux *et al.*, 1974; Perry and Wolff, 1974; Korenberg and Freedlender, 1974).

1. Different Types of Bands Observed

a. R-Bands. Continuous treatment for the final 3–15 hours generally modifies all or part of the Q-bands in such a way that the chromosomes display R bands (Fig. 7). This modification follows a very characteristic cycle which is a function of the treatment period. For nearly 3 hours, only some Q-bands are affected, and they show considerable elongation. For approximately 5–7 hours, nearly all of the Q-bands are modified and show great elongation. Their color properties, in particular with respect to acridine orange, are significantly modified (red staining). Beyond 7 hours and up to 13 or 15 hours, some R-bands, as well as all of the Q-bands, are affected. The elongation of these R-bands is generally little or nonexistent, but their red coloration with acridine orange is highly characteristic.

There is a well-established sequence in the order of modification of chromatid segments (Dutrillaux and Fosse, 1974; Dutrillaux, 1975). To the extent that BUdR is involved in S phase, by incorporation within the DNA molecule, it is obvious that this study will permit definition of the precise chronology for replication of the various chromosome bands (Kim *et al.*, 1975; Grzeschik *et al.*, 1975; Dutrillaux *et al.*, 1976b).

b. Q-Bands. Discontinuous treatment, from the fifteenth to the eleventh hour prior to fixation, for example, can modify R-bands, which may be sparse and not elongated but may have modified coloration with Hoechst 33258 fluorochrome (Latt, 1973), with acridine orange (Dutrillaux, 1975), or even with the Giemsa solution, with or without prior heat treatment (Grzeschik *et al.*, 1975; Viegas-Pequignot and Dutrillaux, 1976). The result is an extremely contrasted Q- or G-banding, accompanied by staining of the centromere regions (Fig. 8). On the average, the number of bands observed is very high.

One can conclude that the R-bands, which are selectively modified in this case, furnish an example of structures in which DNA replication is very early. Q-bands, on the other hand, have a late DNA replication. Banding of the Q type has also been described after staining with Hoechst product 33258 and BUdR treatment under somewhat similar conditions (Latt, 1973).

c. Intermediate Bands. When treatment is applied from the ninth to the thirteenth hour prior to fixation, the metaphases often display patterns that are intermediate between R and Q (Dutrillaux, 1975a). Certain chromosomes have R-bands (Fig. 9), while others have Q-bands. Others have R-bands on one segment and Q-bands on another. For X chromosomes, one may bear R-bands (the early replicating X chromosome) and another may bear Q-bands (the late replicating X chromosome).

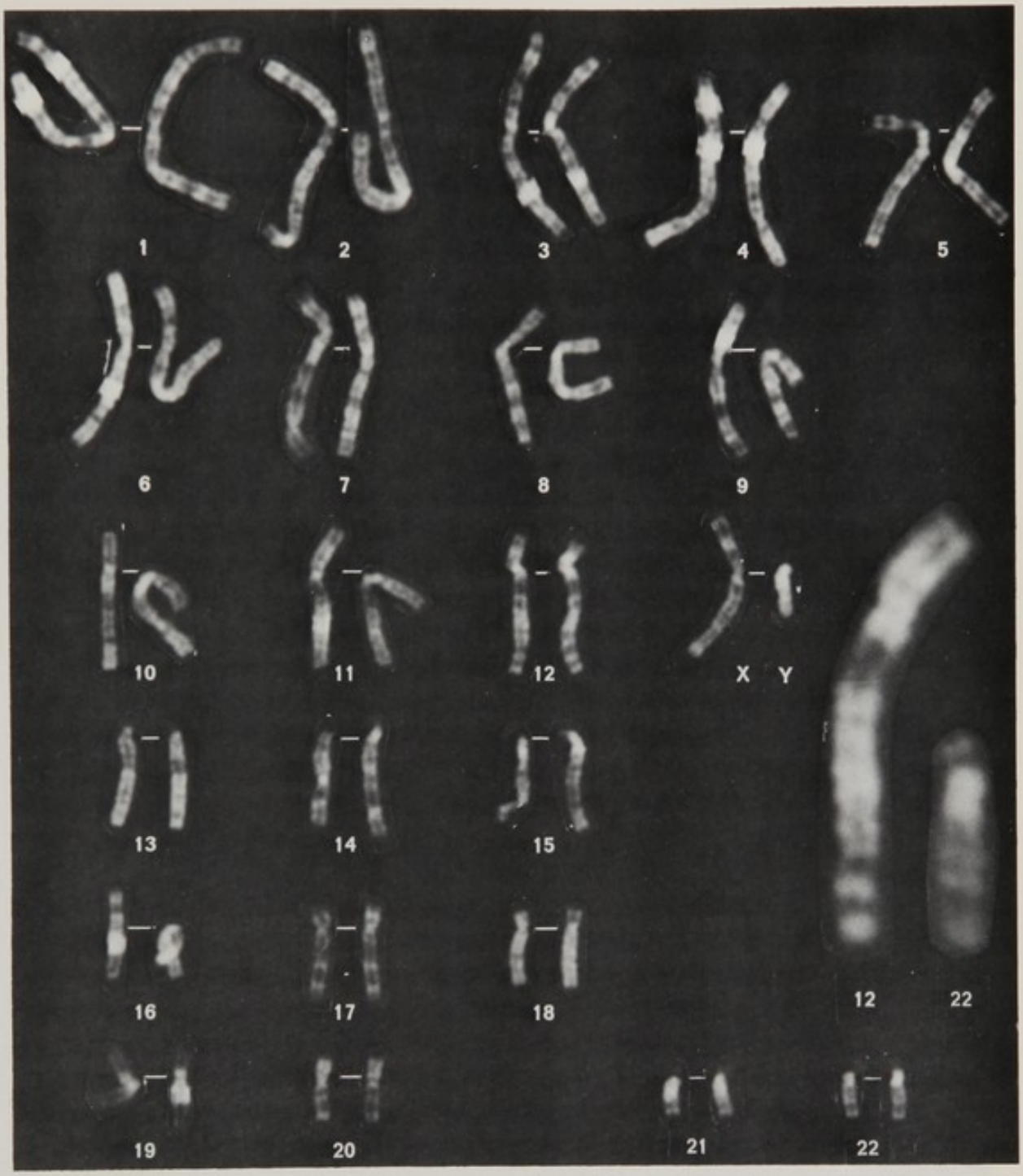

Fig. 8. Q-bands observed after discontinuous BUdR treatment and staining with acridine orange. In the lower right side, enlargements of chromosomes 12 and 22 can be seen.

This indicates that toward the middle of S phase, the replication times for certain R-bands and those for certain Q-bands are very close. Indeed, it is possible that they are even superimposed.

d. H-Bands. In cases of discontinuous treatment, such as one uses for obtaining Q-bands, a portion of the mitoses is banded in a very distinct manner.

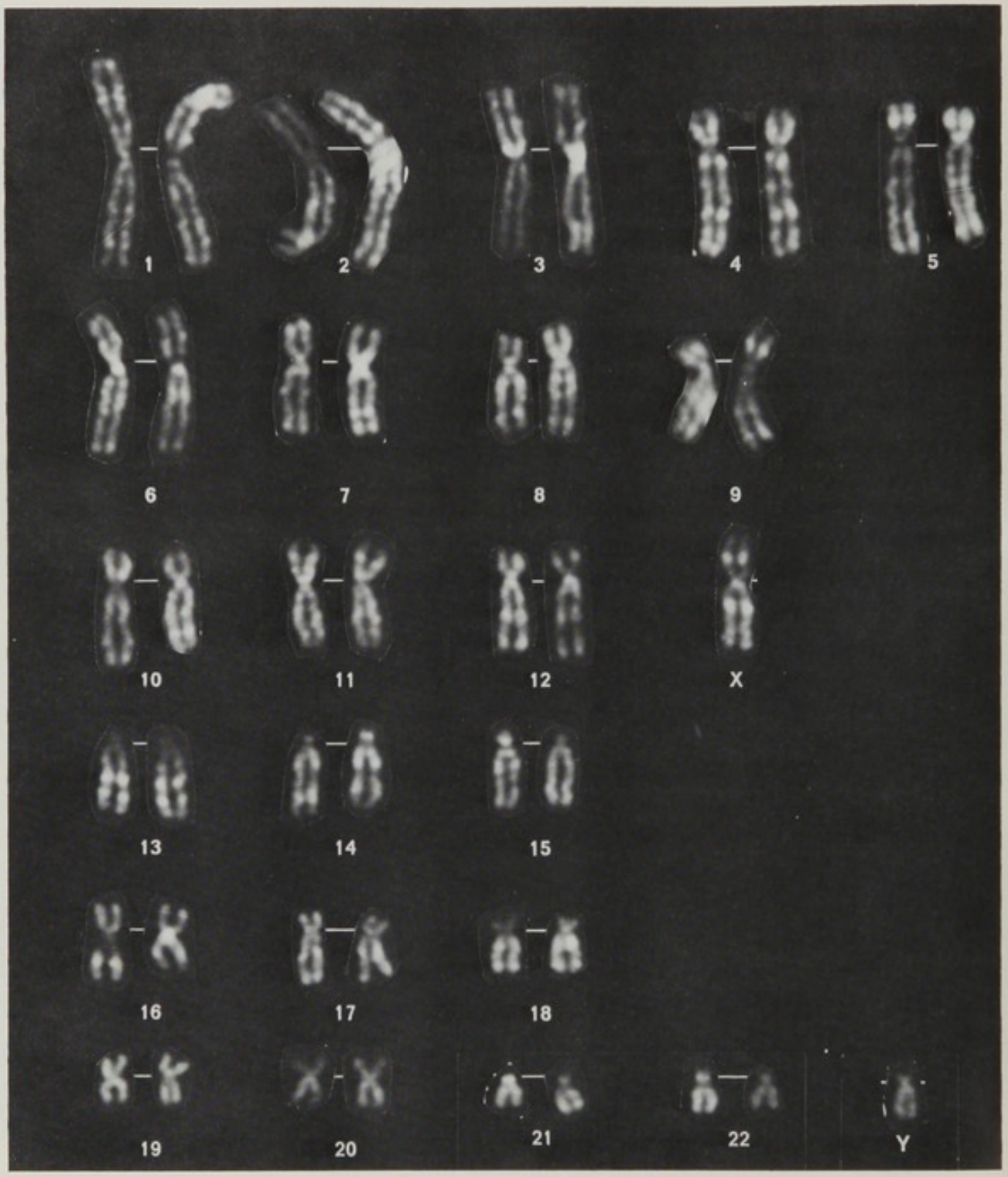

Fig. 9. Intermediate banding between R- and Q-bands.

except for certain regions. The latter probably correspond to a particular type of heterochromatin and are stained green when acridine orange is used (Fig. 10).

Localization of this banding pattern is very interesting for chromosome 2 in that we know that tens of millions of years ago, chromosomes corresponding to an ancestral primate species were acrocentric (Dutrillaux, 1975b) and were probably heterochromatin bearers.

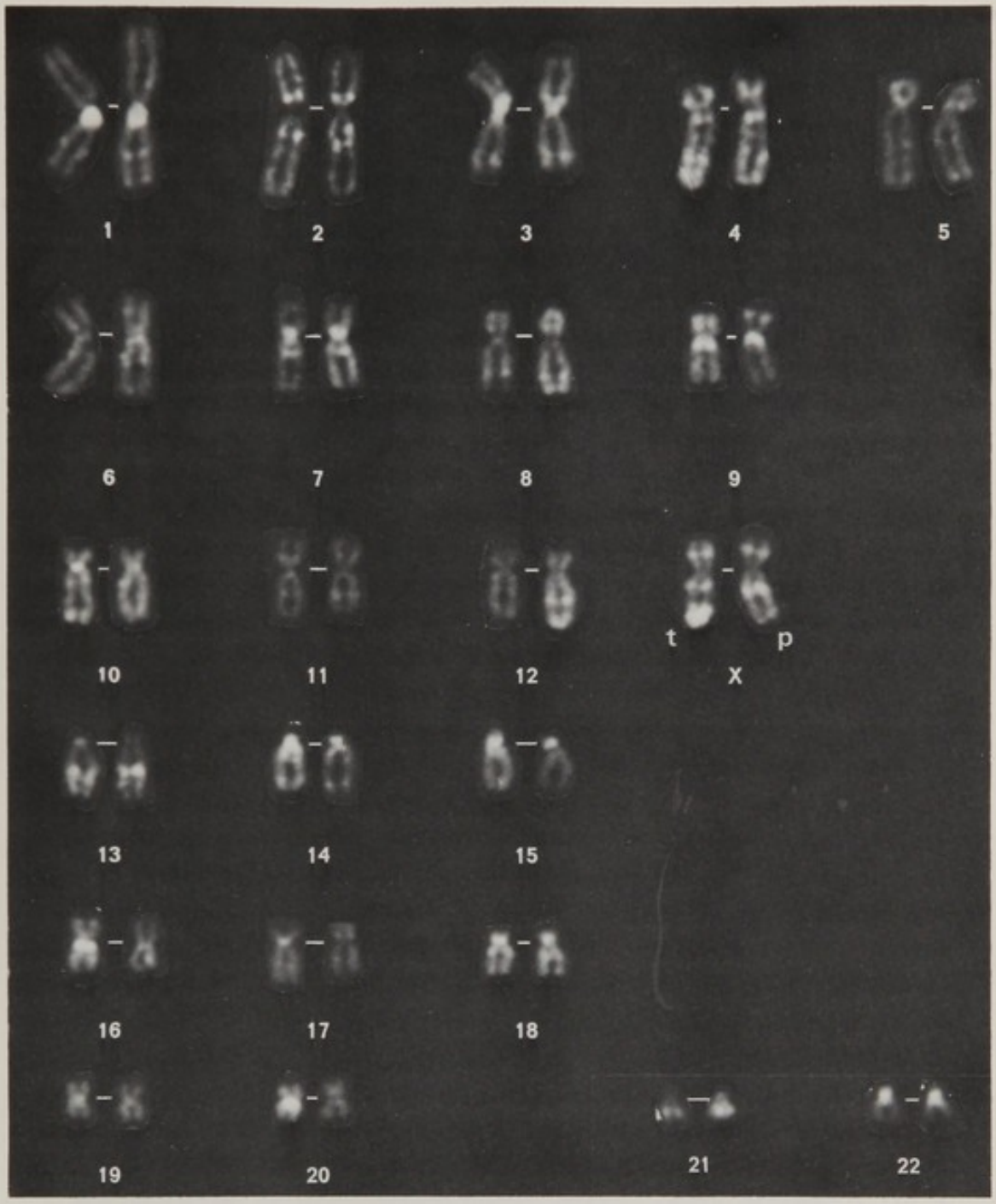

Fig. 10. Staining for a special kind of heterochromatin (H-bands). The late-replicating X chromosome is highly fluorescent. Also note the presence of heterochromatin in chromosome 2.

e. Chromatid Asymmetry with R-Banding. This chromosomal pattern appears after continuous treatment for the last 17–24 hours of culture. For each chromosome, one of the chromatids appears normal; there is no elongation, and there is a homogeneous green fluorescence. The other chromatid by contrast, is elongated due to the action of BUdR and has a somewhat orange fluorescence (Fig. 11). This chromatid therefore has an R-banding pattern. These modifica-

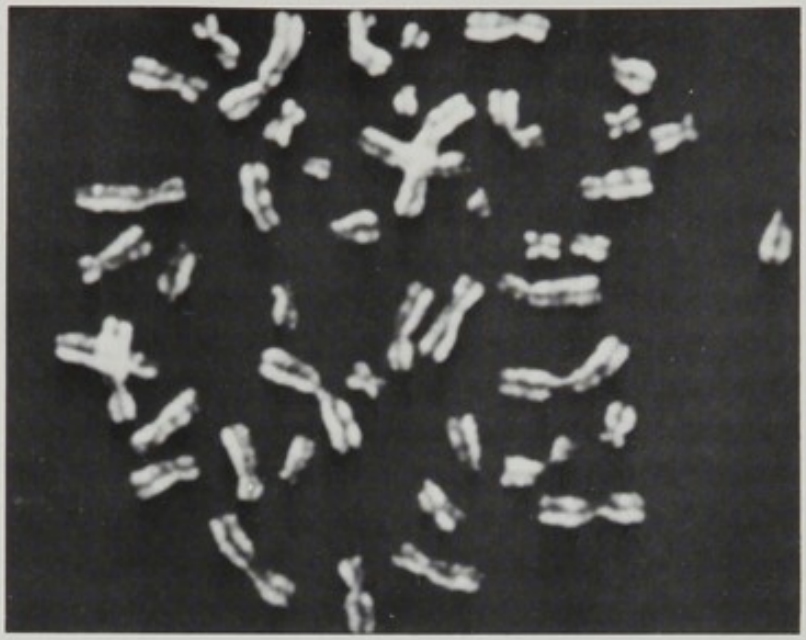

Fig. 11. Chromatid asymmetry, with R-banding of the longer chromatid.

tions probably correspond to those which were originally described by Zakharov and Egolina (1972).

f. Chromatid Asymmetry without Banding. More prolonged treatment will fully modify one of the chromatids. The chromosomes then become perceptibly asymmetrical and convoluted. The longer chromatid lies in a circle whose radius is larger than that of the shorter chromatid.

Several techniques have been described, with use of either Hoechst product 33258 (Latt, 1973) after treatment or of acridine orange. There can be either double staining and a heat treatment (Perry and Wolff, 1974) or a simple heat treatment and Giemsa staining (Korenberg and Freedlender, 1974).

The shortest chromatid (the one least modified by BUdR) is, therefore, the most deeply stained one. The longest chromatid is either less deeply stained or has a somewhat orange fluorescence (with acridine orange staining).

Chromatid exchanges: This characteristic of the two sister chromatids permits observation of exchanges in both chromatids (Fig. 12). Unfortunately, with BUdR inducing the occurrence of these exchanges and with their frequency being proportional to the amount of BUdR used, the question of whether chromatid exchanges exist without treatment of any kind still remains unanswered. In spite of this, the method has proved useful for study of other factors which may also be capable of inducing exchanges. In this way, for example, it has been shown that in Bloom syndrome their frequency is greatly increased (Chaganti *et al.*, 1974). It also appears that exchanges which have been observed may represent exchanges between DNA strands that emerge during the S phase.

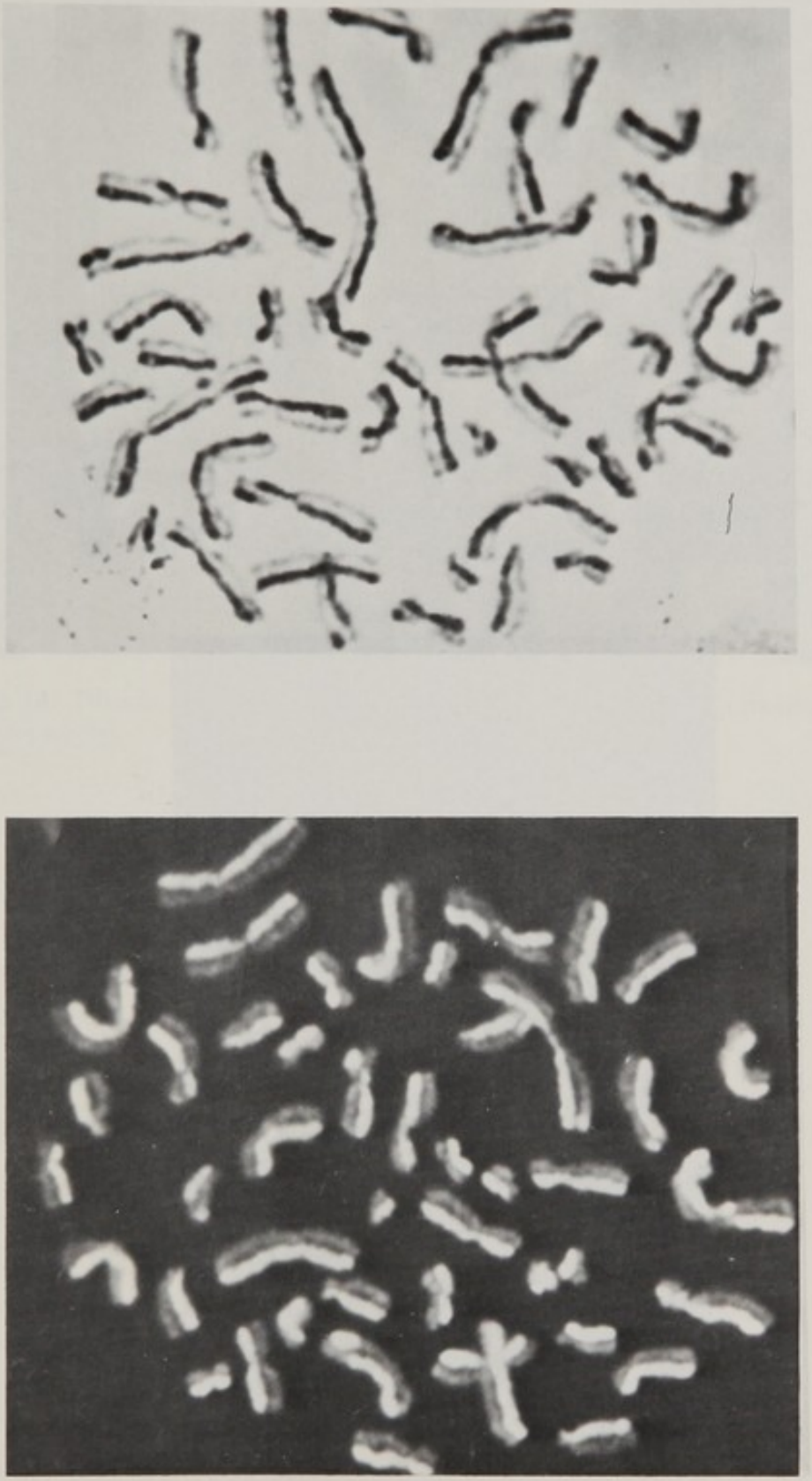

Fig. 12. Chromatid asymmetry and chromatid exchanges: (left) acridine orange staining; (right) staining with Giemsa.

g. Chromatid Asymmetry with Q-Banding. This has been observed with discontinuous treatment, such as a 24-hour BUdR treatment followed by a 36-hour treatment with a concentrated dose of thymidine (Dutrillaux, 1975c). In this case the longest chromatid is homogeneous, and it is stained red by acridine orange, whereas the shortest chromatid has the same Q-bands described in Section III,A,1,a (Fig. 13). This technique is undoubtedly one of the most revealing ones, since it permits combined recognition of Q-bands, chromatid exchanges and their relation to Q-bands, and the late replicating X chromosome which is highly fluorescent.

h. Chromatid Asymmetry with H-Banding. This type of marking, albeit less frequent, is obtained under the same conditions as the preceding banding. It is characterized by the presence of an elongated chromatid, which has a homogeneous red coloration with acridine orange, and a chromatid that is marked with H-bands similar to those described in Section III,A,1,d (Fig. 14).

i. Chromatid Asymmetry and R-Banding of the Two Chromatids. A double BUdR treatment permits one to dissociate asymmetry and segmentation of chromatids entirely. The first treatment is with a low concentration of BUdR (1

Fig. 13. Chromatid asymmetry and Q-banding of the shorter chromatid.

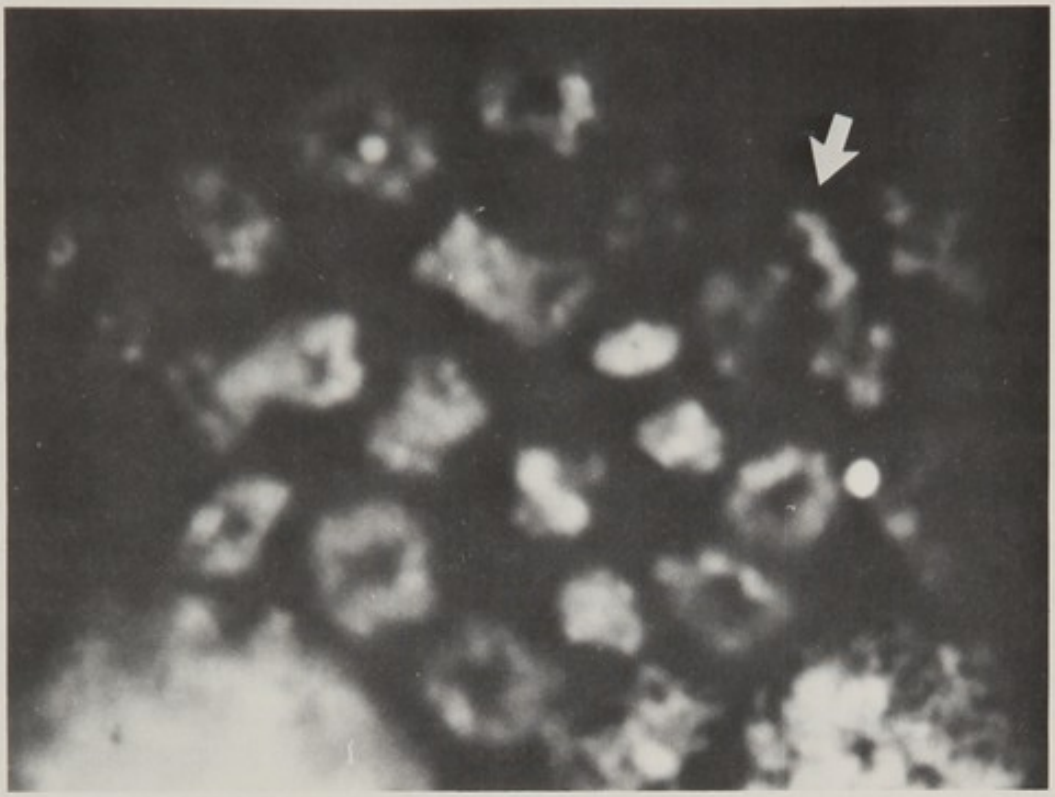

Fig. 14. T-bands of a primary spermatocyte from a subject having a translocation (14q; 22q) (see arrow).

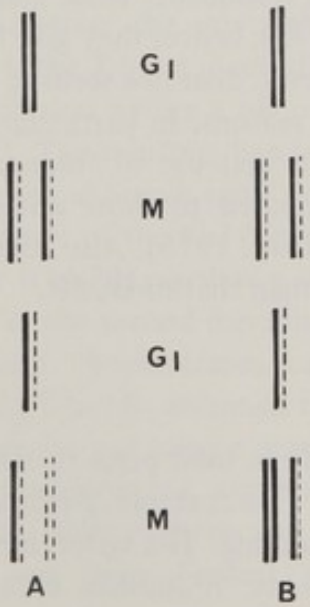

Fig. 15. Diagram showing incorporation of BUdR during the last two cell cycles (A) and during the penultimate cell cycle (B) only. Dotted line = DNA having incorporated BUdR.

μgm/ml) for the last 24–30 hours. The second is treatment with a concentrated quantity of BUdR (100 μgm) for the last 7 hours (Dutrillaux *et al.*, 1976a). In this case, the chromatids, which are of unequal length, both have an R-banding pattern similar to that which was described in Section III,A,1,a (Fig. 15).

2. Conclusion

The methods which involve a preliminary treatment with BUdR have proved particularly interesting for several reasons. At the practical level, they are generally simple to execute and they furnish extremely consistent results. Also, the banding which they produce are of excellent quality, with very good contrast between bands, especially after staining with acridine orange. Finally, the information which they furnish (banding, chromatid exchanges, recognition of heterochromatic segments) is considerable. At the theoretical level, they permit one to deal with fundamental problems which concern chromosome replication and its relationships with R- and Q-bands or with chromatid exchanges. Finally, at the rate at which most of these techniques have been discovered recently, there are excellent possibilities that further developments will emerge in the near future.

B. Treatment with Actinomycin D

It has been known for a very long time that actinomycin D (AMD) is capable, when it is introduced into a culture medium, of producing chromosome segmentations and mutations. More recently, it has been found that with low dosages, on the order of several micrograms, chromosomes develop elongation zones as they do after BUdR treatment. These zones, however, exist at the level of the R-bands (Hsu *et al.*, 1973); hence, they give the appearance of G-bands.

The AMD treatment, however, does not seem to offer as bright a future as the BUdR treatment for several reasons. In particular, the staining qualities of the chromatids are less altered so that use of fluorochromes hardly produces any interesting results when compared to those obtained with a Giemsa solution (Viegas-Pequignot and Dutrillaux, 1976). Also, the toxicity of AMD means that its use requires far more care than that of BUdR.

C. Other Treatments

Other products have also been used prior to stabilizing cells in an attempt to obtain chromosome banding. For example, β-mercaptoethanol (Zakharov *et al.*, 1971) appears to induce G-banding. The latter, however, is of mediocre quality. and, as the preceding methods, it induces elongation of certain chromatid segments. Acridine orange has also been used for pretreatment (Hsu *et al.*, 1973;

Dutrillaux and Lejeune, 1975). It does not modify chromatid condensation, but it does modify staining qualities with respect to Giemsa solutions. R-bands become less chromophilic, and a G-banding appears.

IV. CHROMOSOME BANDING OF VARIOUS TYPES OF CELLS

All of the methods which we have previously described have been developed primarily using lymphocyte cultures. Without exception, they are also applicable to fibroblast cultures, but the quality of banding is usually somewhat inferior, probably because of the initial quality of the preparations. BUdR treatments are also possible, but fibroblasts do not react as well as lymphocytes.

Amniotic cells are also suitable for chromosome banding, at least with respect to the results obtained with treatment of stabilized preparations. Marrow cells furnish results, but the quality is generally poor. In our opinion, it is appropriate to perform a protracted stabilization of the cells prior to using banding methods. Indeed, the greatest difficulties occur in analysis of germinative cells, and we shall discuss this topic further.

The first results were obtained by Caspersson *et al.* (1971). They were able to classify the various bivalents of the first metaphase in relation to their Q- and C-bands. These results remained somewhat isolated, and very few authors were able to furnish, for example, clear identification of structural changes in meiotic chromosomes by these techniques (Hulten, 1974; Chandley and Fletcher, 1973; Dutrillaux and Gueguen, 1975).

With the exception of C-banding, which can be performed with relative simplicity but is not very informative, the only technique which has furnished us with highly consistent results is the T-band method (treatment of recent preparations at 87°C in an Earle's solution having a pH of 5.1 for nearly an hour, and staining with acridine orange). The resulting bands are relatively comparable to the T-bands described previously, although some ambiguities continue to exist in terms of classifying chromosomes of the 6–12 group. Recognition of translocations is possible, for example t(14-22), as shown in Fig. 14. Recognition can be for either the first metaphase or the second metaphase.

As for spermatogonia, their chromosomes can be identified by Q-bands (Hulten, 1974), and C-, R-, and T-bands, although the same difficulties exist.

Banding of prophase I in the pachytene stage would be of the greatest interest, since the bivalents are elongated, especially at the narrow synapse, so that inversion loops and other abnormalities can be detected. However, difficulties remain numerous, and only limited results have been obtained (Luciani *et al.*, 1975).

V. ORIGIN OF CHROMOSOMAL BANDS

It does not fall within the scope of this section to develop the theme of the origin of bands, in relation to the chemical structure of chromosomes. Other articles have been dedicated to that subject. Nonetheless, after our examination of chromosome banding techniques, it is useful to recall the principal features of different types of bands, and, in particular, the conditions for their appearance and disappearance. Finally, by analysis of several anthropoid primates, we shall see how it is possible to trace the origin of certain structures, by localization or by their characteristics, going back over several millions of years.

A. Characteristics of the Principal Banding Patterns

1. Banding of Stabilized Preparations

Chromosome bands, properly speaking, fall into two groups, Q- or G-bands and R-bands. These are distinguished from one another both by location and by the way in which they emerge.

In order to summarize what has been presented previously, it can be considered that Q- or G-positive bands are structures which comprise nearly 50% of the chromatids. They are recognized by their more intense chromophilic qualities, especially with respect to quinacrine mustard, and also with respect to Giemsa's solution, acridine orange, and Hoechst derivative 33258. Their resistance to heat treatment as well as to proteolytic urea enzyme digestion and to detergents appears relatively high.

Finally, their DNA usually undergoes a delayed replication (Ganner and Evans, 1971; Dutrillaux, 1975a).

The R-positive bands, which comprise nearly 50% of the chromatids are located precisely opposite the Q- or G-bands. Chromophilic qualities are weak, so that one can only observe them after specific modification or destruction of G- or Q-bands. Their resistance to heat treatment is greater, especially with respect to bands located close to certain telomeres (T-bands). Resistance to proteolytic enzymes, urea, and detergents, however, is low.

Their DNA is replicated early and appears to be scaled according to placement of bands along the entire early S phase. In fact, even though the heterogeneity of each group of structures is obvious (for example, one can progressively eliminate R-bands in order to obtain T-bands), it should be possible to differentiate Q- or G-bands and R-bands according to precise biochemical features, and to replication time (Dutrillaux *et al.*, 1976b). This would be easy, except that one is confronted with the problem of the particular chromosome structures, which essentially consist of DNA and various proteins in close association with one another and with presumably interdependent composition.

Thus, any treatment or staining procedure, no matter how specific, will always be dependent upon those two components. Hence, it is not surprising that over the years and according to individual experimenters, interpretations of the origin of banding have varied widely.

We cite the following arguments in favor of the predominant role of DNA composition: (1) greater fluorescence of nucleotides having a high AT content (Q-positive bands are segments that are rich in AT); (2) greater resistance to DNA denaturation according to GC content (R-bands are segments that are rich in GC); (3) the tendency toward late replication of segments with high AT content (Q-positive bands and especially heterochromatic regions are rich in AT).

However, the following factors considerably reduce the impact of these arguments: (1) differences observed in Q fluorescence are of a much broader range than differences of concentration in AT and GC bases on chromosomes (Comings and Avelino, 1974); (2) heat treatment specifically masks the R-bands or G-bands, and it is possible to pass from one type of banding to another. This implies, for example, that treatment which induces G-bands does not necessarily denature the DNA of R-bands, or vice versa (Dutrillaux and Covic, 1974; Dutrillaux, 1975b); (3) in terms of chromatophilic qualities as a function of concentration of AT or GC bases, heterochromatin of human chromosomes 1, 9, and 16 which contains a satellite DNA rich in AT should release an intense Q fluorescence. In reality, however, it is practically nonfluorescent; (4) finally, if one considers proteolytic enzymes as having an important effect on banding (Dutrillaux *et al.*, 1971), it is highly probable that, like DNA, proteins play an important role. For example, stabilization of staining substances could be largely determined according to the type of DNA-protein association, and the various treatments would be particularly affected by modification of that association.

Heat treatment could modify the structure or permeability of proteins in such a way as to prevent the staining substance from attaching itself to the underlying DNA. Then, by elimination of these proteins, it could furnish the chromophilic qualities to the corresponding chromatid segments. In this way, the same structure could initially be chromophilic and then chromphobic, finally becoming chromophilic again (Dutrillaux and Covic, 1974; Comings and Avelino, 1974).

2. Banding Induced by BUdR Treatment

a. R- and Q-Bands. BUdR, as a replacement for thymidine, results in a delay of Q-positive band formation. Since the pattern observed corresponds particularly to a modification of Q-bands (R-banding), one could conclude that Q-positive bands are particularly rich in AT. In fact, we noted previously that all types of bandings, R and Q in particular, can be induced simply by varying the incorporation period relative to this phase. Thus, it is necessary to deduce that

DNA composition and, in particular, its high AT content do not directly influence R- and Q-bands, at least in the BUdR method (Dutrillaux, 1975a).

b. Asymmetries. We noted that chromatid asymmetries appear when BUdR is incorporated starting with the penultimate cellular cycle (Zakharov and Egolina, 1972). However, the surprising fact is that incorporation during the final cycle is entirely optional (Dutrillaux *et al.*, 1975). In other words, when incorporation occurs only during the penultimate S phase, chromosomes have the same appearance as when incorporation takes place during the last two S phases. In the first case, the chromatid which has a single DNA strand replaced is the one which is modified, whereas it is not modified in the second case (Fig. 15). This leads one to believe that chromatid structure does not depend directly on DNA composition during metaphase. It would depend directly on interphase DNA, particularly during phase G_1. At that particular point, the two situations described previously are identical, with each chromosome having a single chromatid and possessing a normal DNA strand and a substituted strand (Dutrillaux *et al.*, 1975).

B. Phylogenetic Origin of Certain Chromosome Structures

Study of anthropoid primates shows numerous similarities among their chromsomes and those of man (de Grouchy *et al.*, 1972; Turleau *et al.*, 1972; Pearson, 1973; Lejeune *et al.*, 1973; Dutrillaux *et al.*, 1973b, 1975).

Precise comparative analysis permits us to propose a sequence of chromosomal changes that have evolved since the last common ancestor with anthropoids and, in this way, to reconstruct the karyotype of species which disappeared millions of years ago (Dutrillaux, 1975b). For a certain number of chromosomes, the particular features found in humans can be explained by unusual chromosomal changes. Let us consider, for example, chromosomes 2, 9, and 11.

1. Chromosome 2

Figure 16 shows the sequence of changes which affected the components that are the source of chromosome 2 in human beings. The chromosomes which represent successive ancestral stages are still found in orangutans, gorillas, and chimpanzees.

In the common ancestor, the components were acrocentric, and heterochromatin was located on the short arm. This has been demonstrated with the help of the G_{11} method. The components are represented in the orangutan. In order to reach the "gorilla" stage, a pericentric inversion which created the essential structure of the long arm of chromosome 2 in human beings (2q) formed a submetacentric chromosome whose heterochromatin, certainly, was displaced, along with the centromere, toward the interior portion of the chro-

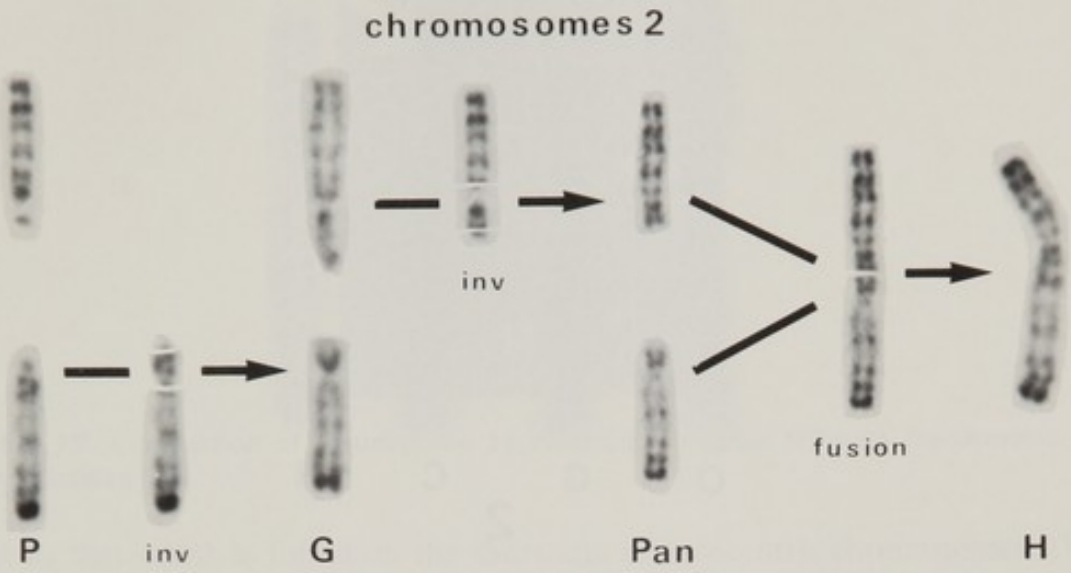

Fig. 16. Chromosome 2 from man and equivalent chromosomes from anthropoid primates, with changes that have occurred since the common ancestor, whose chromosomes can be assumed to have been the same as those of the orangutan. P = orangutan, G = gorilla, Pan = chimpanzee, H = human.

mosome. The heterochromatin is no longer subject to staining with the G_{11} technique.

To reach the "chimpanzee" stage, the chromosome just described remained unchanged, but the components of the short arm in chromosome 2 in humans (2p) also underwent a pericentric inversion. Along with the centromere, the heterochromatin of the short arm was displaced, but it retained its staining qualities for the G_{11} method in present-day chimpanzees. For the "human" stage, a fusion of the two preceding components had to occur at the telomere of the short arm. As we have demonstrated (Lejeune *et al.*, 1973), that change induced a deactivation of one of the two centromeres. However, in some cases, there is still a gap at the point of fusion among human beings, along with anomalies of the farthest segment of the long arm.

If this description is accurate, one should be able to distinguish heterochromatin both on the short arm of chromosome 2 in human beings (in the same area as for chimpanzees) and on the long arm (near the location of the original centromere). This has become possible with BUdR treatment techniques (Figs. 10 and 17).

2. Chromosome 9

Among chromosomes which contain a secondary constriction, chromosome 9 in human beings is distinguished by features such as the particular composition of its satellite DNA and a pronounced staining with the G_{11} method (Bobrow *et al.*, 1972; Gagne and Laberge, 1972).

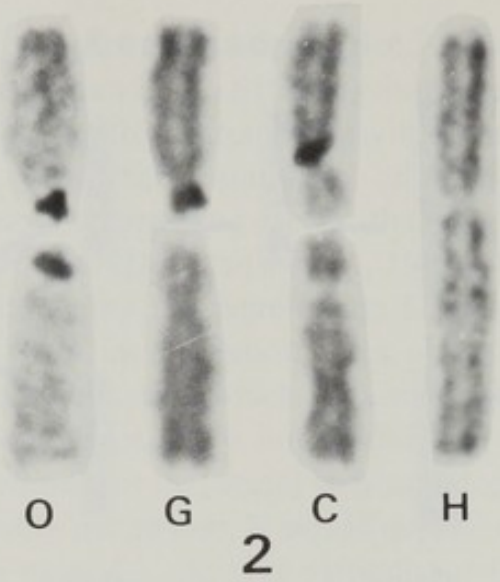

Fig. 17. Comparison of heterochromatin from chromosome 2 in humans, with that of equivalent components for anthropoid primates. Heterochromatin staining is only identifiable in humans after BUdR treatment. O = orangutan, G = gorilla, C = chimpanzee, H = human.

Analysis of anthropoids shows that the equivalent element is acrocentric in orangutans and gorillas (Fig. 18), whereas it is submetacentric in chimpanzees, but differs from chromosome 9 in humans. Since one can reconstruct chromosome 9 of the chimpanzee by transference and that of humans by pericentric inversion on the basis of chromosome 9 from orangutans and gorillas, it appears that the latter still possess the ancestral component. In this case, the secondary constriction of chromosome 9 in man would correspond to a substance equiva-

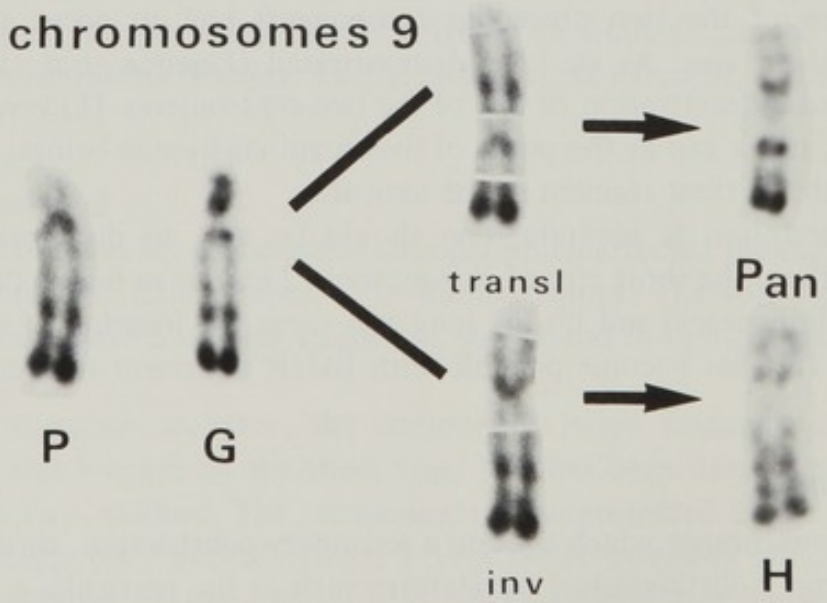

Fig. 18. Evolution of chromosome 9. P = orangutan, G = gorilla, Pan = chimpanzee, H = human.

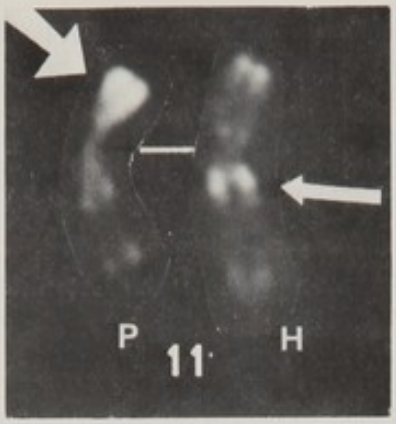

Fig. 19. Comparison of chromosome 11 from an orangutan (P) with chromosome 11 from a human (H).

lent to that which is found on the short arm of acrocentric chromosomes. This would explain, for example, why the staining properties of these different segments are very similar.

3. Chromosome 11

T-bands are characteristically located at the ends of certain chromatids (Fig. 6). There is, however, one exception: chromosome 11 in man possesses a T-band within the proximal one-third of its long arm, in addition to that found at the end of the short arm.

The equivalent chromosomes of the gorilla and the chimpanzee possess this same unusual banding pattern. On the other hand, orangutans do not have such patterns, and the T-band of the short arm is much larger. Thus, it seems probable that the orangutan still possesses the ancestral chromosome, with a terminal T-band, and that a transference of a large portion of the band occurred prior to the emergence of the direct ancestors of the other species (Fig. 19).

Since the breaking capabilities of the end portions of chromosomes are well known in cases of aberrations (Lejeune *et al.*, 1973; Jacobs *et al.*, 1974), this could also furnish an explanation of the high sensitivity of the intercalated T-band in chromosome 11.

Still other examples could demonstrate the changes which chromosomes have undergone during evolution, and a detailed description has been provided (Dutrillaux, 1975b). The purpose of revealing these examples is to attempt to provide an explanation for some of the frequent abnormalities which affect certain chromosome segments in man.

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8

Banding Patterns, Chromosome Polymorphism, and Primate Evolution

P. L. PEARSON

I. INTRODUCTION

It is a salutary fact that the first correct counting of the chromosome number of the chimpanzee (*Pan troglodytes*) predated that of the human by approximately 15 years (Yeager *et al.*, 1940). In the late 1950's and early 1960's, interest in the karyology of primates was rearoused by the application of cytogenetic techniques developed primarily for investigating the human karyotype (Chu and Bender, 1962; Chiarelli, 1962; Hamerton *et al.*, 1963). The majority of these early studies were concerned with making comparisons with the human karyotype, and it is not surprising that more attention was paid to the great apes and their close relatives than to other primate groups. These studies established that the chromosome number in the chimpanzee, gorilla and

orangutan was 48 and not 46 as in man, and on the basis of size and shape parameters, attempted to compare individual human chromosomes to their supposed human counterparts. The general conclusion was that the chimpanzee karyotype most closely resembled that of man, and that the orangutan, although possessing the same chromosome number as the chimpanzee and gorilla, appeared to have a very different karyotype. Despite the limitations of analysis based entirely on size and shape parameters, attempts were made to assess how the 46 chromosome karyotype of the human could have been derived from an ancestral line with 48 chromosomes, and deductions were made which went far beyond reasonable levels permitted by morphological criteria alone (Chiarelli, 1972). The introduction of differential staining techniques during the last few years has permitted a detailed reinvestigation of the relationship between the human karyotype and that of other primates. It is the purpose of this chapter to review this evidence and to compare it with the information now emerging from related fields, such as gene mapping, and to study the distribution of satellite DNA. Finally, the implications of this evidence in assessing the importance of chromosomes as indicators of evolution will be discussed.

II. CHROMOSOME VARIATION DEFINED BY BANDING TECHNIQUES

The discovery that quinacrine mustard and related compounds produced differential staining along chromosomes and, in particular, a bright fluorescent segment on the distal half of the human Y chromosome stimulated research for the presence of similar differentiation in the chromosomes of primates and other mammalian species (Pearson *et al.*, 1971). These investigations showed that intensely fluorescent chromatin was confined in mammals to three primate species, namely, man, chimpanzee and gorilla, and that only man and the gorilla possessed Y chromosomes with intensely fluorescent chromatin. In fact very few animal species of any type have been demonstrated to possess intensely fluorescent chromatin, and we can only surmise that such chromatin arises very infrequently in the course of evolution. Ellison and Barr (1972) and Barr and Ellison (1971) were among the first to demonstrate the presence of a similar quinacrine staining behavior in the chromosomes of various members of *Drosophila*, including *D. melanogaster*, and *Samoaia leonensis*. Using labeling techniques, they surmised that the intensely fluorescent chromatin of the latter species was characterized by "an extremely high, if not exclusive, content of adenine and thymine". This was the first clear indication that quinacrine fluorescence was associated with a particular form of base composition (Miller *et al.*, 1973), and led the way to subsequent studies indicating that quinacrine fluorescence was also probably suppressed in the presence of high G-C con-

centrations and was not entirely explicable in terms of differential binding (see, for example, Weisblum and De Haseth, 1972; Hatfield and West, 1975). In general, these and other studies indicate that intense quinacrine fluorescence is associated with the presence of a particular type of A-T-rich DNA, which is likely to be highly repetitious, and probably contributes, at least in part, to one or more of the human satellite DNA's, including satellites I and IV (Jones *et al.*, 1974; Gosden *et al.*, 1975). Although very little is as yet known about the presence of these and related DNA satellites in primates, the distribution of intensely fluorescent chromatin between species strongly suggests the evolution of one or more satellite DNA's, responsible for the intense quinacrine fluorescence, after the branching off of an ancestral line which resulted in the orangutan, on one side, and man, chimpanzee, and the gorilla on the other (Pearson, 1973) (Fig. 1).

The variation of this type of heterochromatin has been comprehensively described in man (Paris Conference, 1971b) and used for population and family studies (see, for example, Mikelsaar *et al.*, 1973; Geraedts and Pearson, 1974) as well as that described for other forms of constitutive heterochromatin identified as C banding variations (Craig-Holmes *et al.*, 1973; Bobrow *et al.*, 1972) that probably corresponds to the localization of the major human DNA satellites (Jones, 1973; Gosden *et al.*, 1975). A comparison of these variants in human and primate chromosomes will be made in a subsequent section.

Early attempts in comparing the general quinacrine banding pattern of human chromosomes with those of the hominoid apes (Pearson, 1972, 1973; Lin *et al.*,

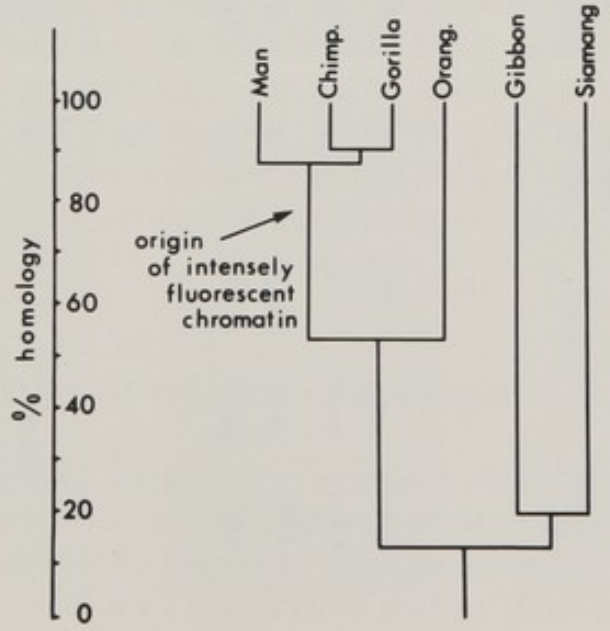

Fig. 1. Evolutionary tree based on chromosome data showing the point of origin of intensely fluorescent chromatin in man and the great apes.

1973) demonstrated that the human chromosomes 1, 3, 5, 6, 7, 8, 10, 11, 13, 14, 15, 19, 20, 21, 22, X, and Y appeared to have direct counterparts in the chimpanzee and to a lesser extent in the gorilla and orangutan, but gave no indication of the way in which the extra two chromosomes involved in the 48 chromosome karyotype of these species were related to the 46 chromosome karyotype of man. It was principally the work of two French groups, using the higher resolution of the Giemsa stained R-banding technique (for example, de Grouchy *et al.*, 1972; Lejeune *et al.*, 1973), that showed that chromosome 2 in man was probably comprised of two acrocentric chromosomes present in the karyotypes of the hominoid apes. It was initially proposed that a centric fusion

Fig. 2. Giemsa-trypsin banded chromosomes of man and the great apes arranged to show the presumed homology with the human karyotype. The human chromosomes are on the left (M) followed by the chimpanzee (C), gorilla (G), and orangutan (O), respectively.

process had taken place (Turleau *et al.*, 1972), but this hypothesis was subsequently modified to one involving a telomeric fusion followed by suppression of activity of one centromere and pericentric inversion (for an extensive review, see Dutrillaux, 1975; see also Chapter 7).

It is interesting to note that two studies based on G- and Giemsa-11 banding and measurement in the chimpanzee (Warburton *et al.*, 1973; Bobrow and Madan, 1973) also implicated the human chromosome 2 as having probably been formed from one or another combination of ancestral primate chromosomes, but failed to find adequate banding similarities to suggest how that had specifically occurred. These differences underline the problems of defining chromosome homology simply in terms of banding morphology and clearly demonstrate the necessity to combine the results of as many techniques as possible, a point followed in the beautifully executed studies of Dutrillaux and colleagues in which they combined the results of at least four techniques. Despite the intensive karyotyping carried out for the last 5 years, there are still certain areas of disagreement over which primate chromosomes are directly homologous to those of man. The homologies shown in Fig. 2 for the chromosomes of man numbered from 1–22, X, Y and their supposed homologues in the chimpanzee, gorilla and orangutan, from left to right respectively, are based on the observations of the author and do not necessarily correspond to those of other authorities. At present the main discrepancies between various workers lie in the specific origin of chromosome 2 in man and those chromosomes in the gorilla and orangutan corresponding to chromosomes 9, 14, 16, 17, and 18 in man.

Most published studies emphasize that pericentric inversion appears to be the most important type of structural difference between individual human chromosomes and those of the great apes. Turleau and de Grouchy (1973) estimated that there are at least eight major pericentric inversion differences between the karyotype of man and the chimpanzee and that, with the exception of chromosome 2, there is no firm evidence for translocation having taken place. The possible importance of pericentric inversion in primate evolution will be discussed later.

III. NOMENCLATURE OF PRIMATE CHROMOSOMES

Several groups have attempted to introduce their own chromosome and banding nomenclature for primate species and, accordingly, a workshop was organized in Lake Placid in December 1974 to determine whether a common proposal could be made for a standardized primate chromosome nomenclature corresponding to the human nomenclature formulated in Paris in 1971. Basically there were two major schools of thought, one of which considered that, since the banding morphology of the hominoid apes was in many ways similar to that

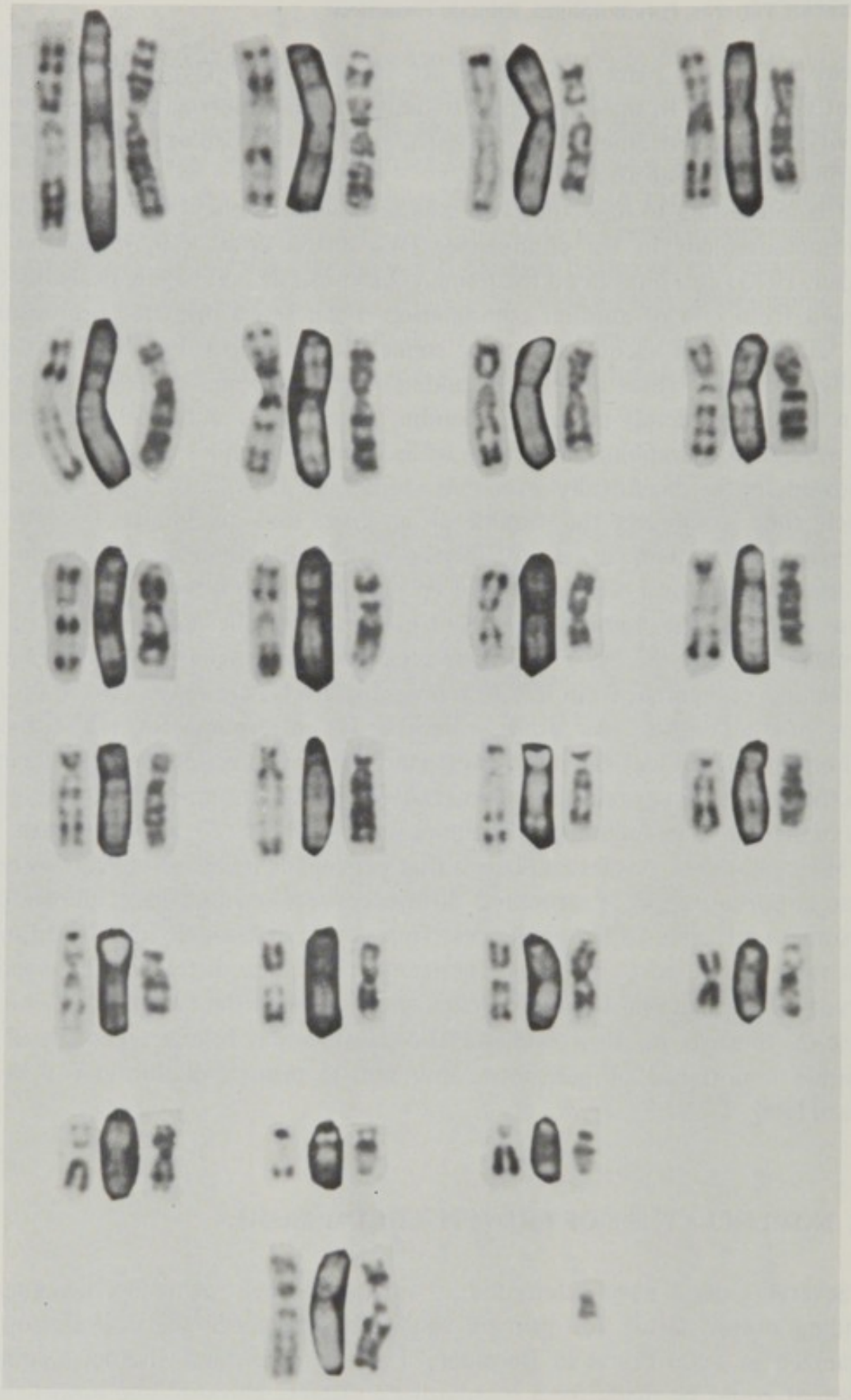

Fig. 3. Composite karyotype of the chimpanzee prepared from R-, Q-, and G-banded chromosomes. The chromosomes are arranged according to length and centromere position. Published with kind permission of the National Foundation. Prepared from material provided by Drs. Dutrillaux, Warburton, and Pearson.

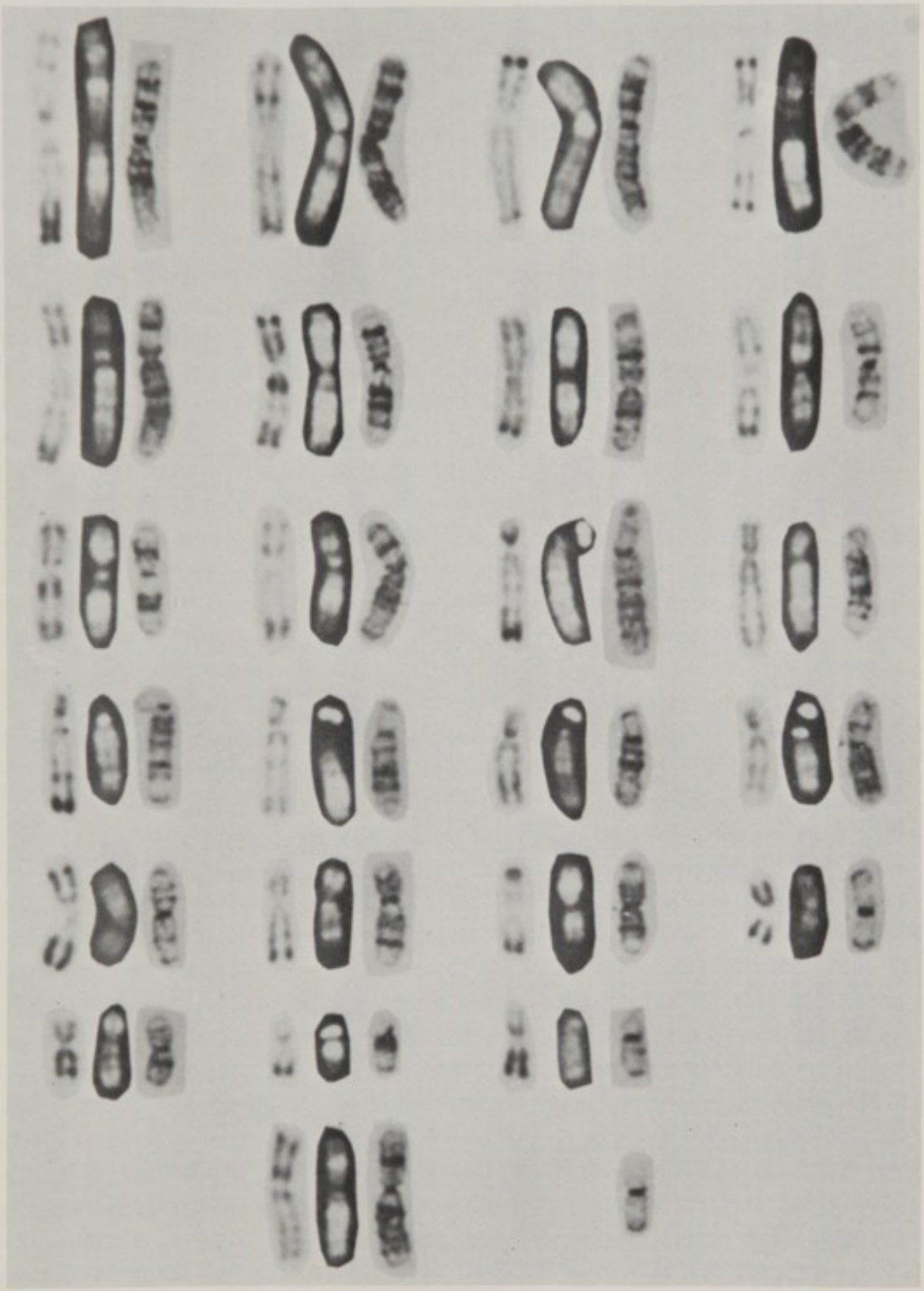

Fig. 4. Composite karyotype of the gorilla prepared from R-, Q-, and G-banded chromosomes. The chromosomes are arranged according to length and centromere position. Published with kind permission of the National Foundation. Prepared from material provided by Drs. Dutrillaux, Warburton, and Pearson.

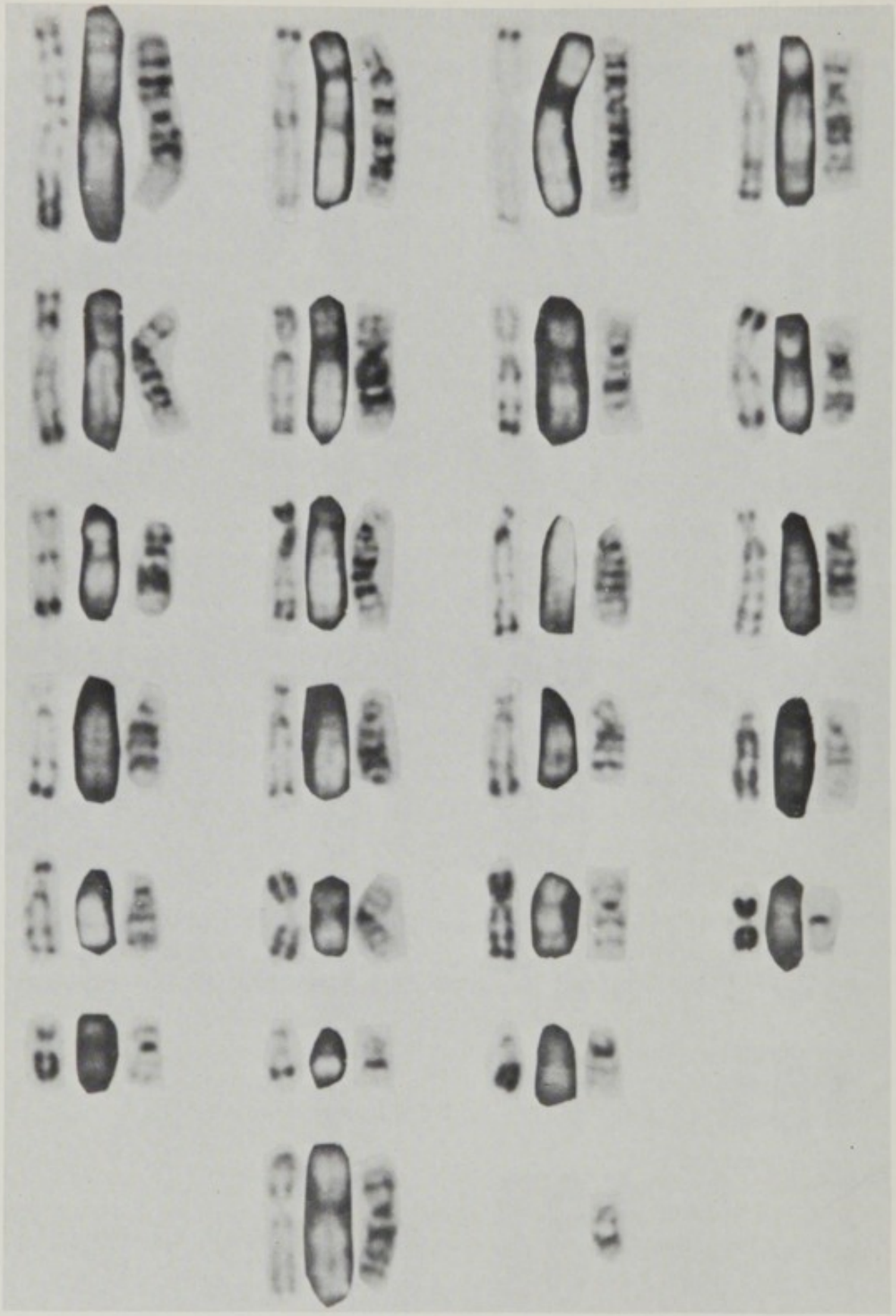

Fig. 5. Composite karyotype of the orangutan prepared from R-, Q-, and G-banded chromosomes. The chromosomes are arranged according to length and centromere position. Published with kind permission of the National Foundation. Prepared from material provided by Drs. Dutrillaux, Warburton, and Pearson.

of man, the hominoid chromosomes should be named directly after their human counterparts as defined by chromosome banding. The other school sponsored the retention of chromosome length and centromere position as the primary classification parameters for two main reasons: the first being that while it might be possible to classify the karyotypes of one or two species according to their supposed human homologies, the homologies could not at present be extended to other species and hence such classification methods were not of potential widespread application; and second, since the supposed homologies were still debatable for some chromosomes and might subsequently have to be modified, that a baseline nomenclature was required which was independent of homology. This latter opinion prevailed and the chromosomes for the chimpanzee, gorilla, and orangutan were arranged and numbered on the basis of chromosome length and centromere position with banding information included (Figs. 3, 4, and 5). Table I gives the human homologies for the various chromosomes of the great apes. The proposed nomenclature has recently been published as a supplement to the Paris report under the auspices of the National Foundation (Paris Conference, 1971b), and includes a banding diagram similar to that provided for the human karyotype in the Paris report in which the bands are also numbered. Figures 6 and 7 are taken from part of this diagram and depict the chromosomes equivalent to human chromosomes 1, 2, 3, and 4 to illustrate certain principles of the nomenclature used. First, each species name has been abbreviated to the first letter of the generic name and the first two letters of the species name. Thus, *Homo sapiens* becomes HSA, *Pan troglodytes* becomes PTR, *Gorilla gorilla* becomes GGO, and *Pongo pygmeaus* becomes PPY, respectively. The chromosomes have been orientated in the diagram to facilitate making comparisons with the human karyotype, and in Fig. 6, for example, chromosomes 12 and 13 of the chimpanzee, designated as PTR 12 and PTR 13, are shown to be homologous to the p and q arms, respectively, of the human chromosome 2. In addition, homology of a band to a particular one present on the corresponding human chromosome is indicated by the band numbers on the right-hand side of each chromosome. It was also recommended in the supplement that homologies could be described in the same way as for structural rearrangements according to the Paris report. For example, the homology between human chromosome 4 and chimpanzee 3 could be described as follows: HSA 4 [PTR, inv. (3) (p1.3,q1.2)] indicating that the main difference between the two chromosomes resides in a pericentric inversion with the break points in p1.3 and q1.2 of chimpanzee chromosome 3. This system has great potential for describing differences between individual chromosomes and has the great advantage that the term within the parentheses can be changed at a later date if new evidence indicates doing so without the problem of having to change the nomenclature of the chromosomes. The system does not of course say where a structural difference has arisen but only that such a difference exists. So in the example described above, there is no

TABLE I
Summary of the Chromosome Homologies of Man and the Great Apes Agreed to in the Supplement to the Paris Report[a]

Man	*Chimpanzee*	*Gorilla*	*Orangutan*
1	1	1	1
2	12, 13	11, 12	11, 12
3	2	2	2
4	3	3	3
5	4	4	4
6	5	5	5
7	6	6	10
8	7	7	6
9	11	–	–
10	8	8	7
11	9	9	8
12	10	10	9
13	14	14	14
14	15	–	–
15	16	15	16
16	18	17	18
17	19	19	–
18	17	16	17
19	20	20	20
20	21	21	21
21	22	22	22
22	23	23	23
X	X	X	X
Y	Y	Y	Y
		13	–
		18	–
			13
			19

[a]Paris Conference (1971a).

way of knowing whether the pericentric inversion difference arose in the human or in the chimpanzee karyotype. However the presence of a similar rearrangement in several species can frequently give some idea of where in the phylogeny of a species group the rearrangement arose. In the case of the pericentric inversion differences found in the Hominoidea, the indications are that they arose mainly in a direct ancestor to man (Dutrillaux, 1975).

To date, comparatively few banding studies have been published on primate species other than the great apes. Pearson (1973) showed, using quinacrine banding, that there were practically no detectable homologies, apart from the X chromosome, between the karyotypes of gibbons and siamangs and those of other hominoid species. These general conclusions have been confirmed recently

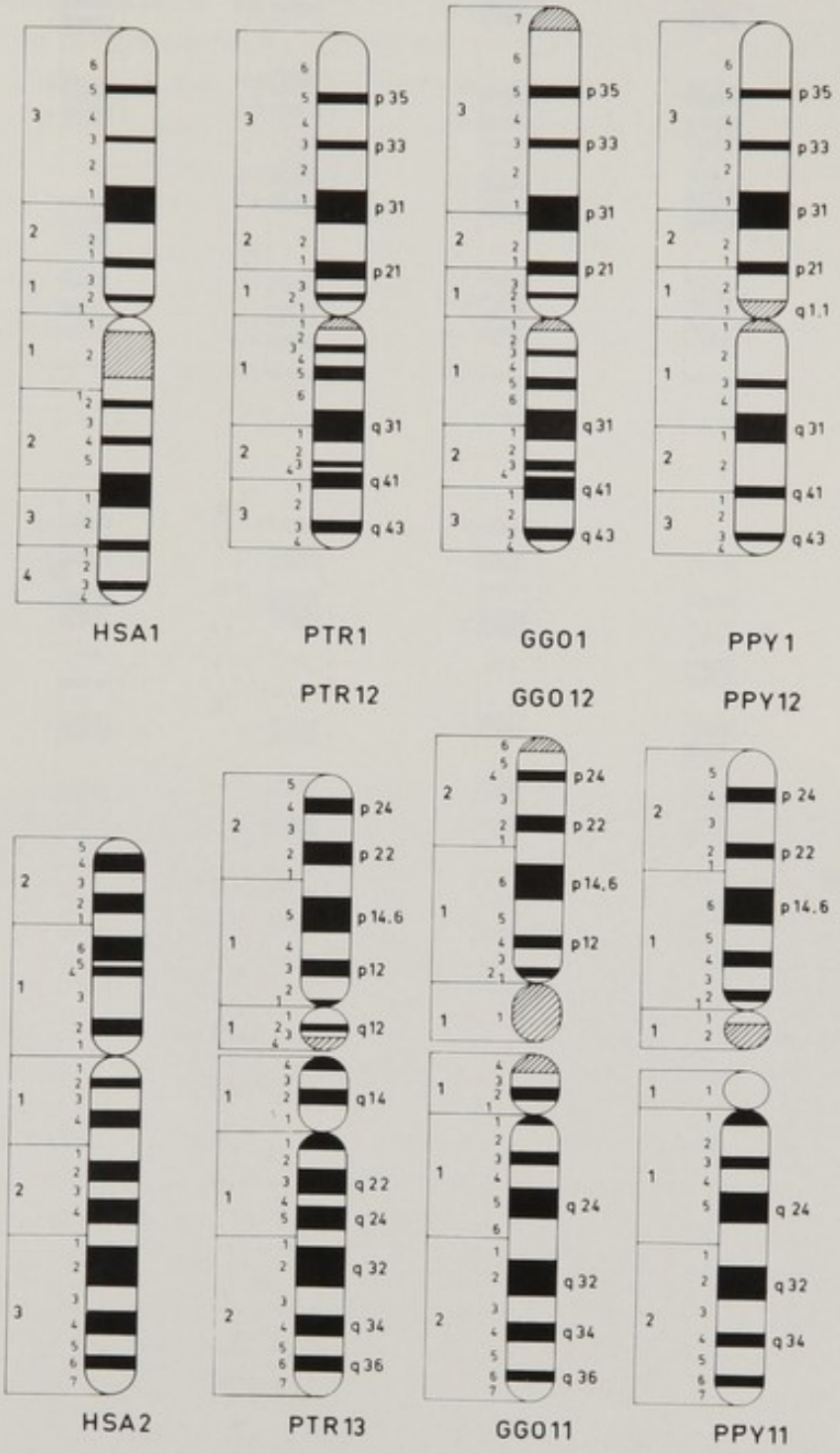

Fig. 6. The band diagrams for human chromosomes 1 and 2 inclusive and their supposed homologues in the great apes. Published with kind permission of the National Foundation.

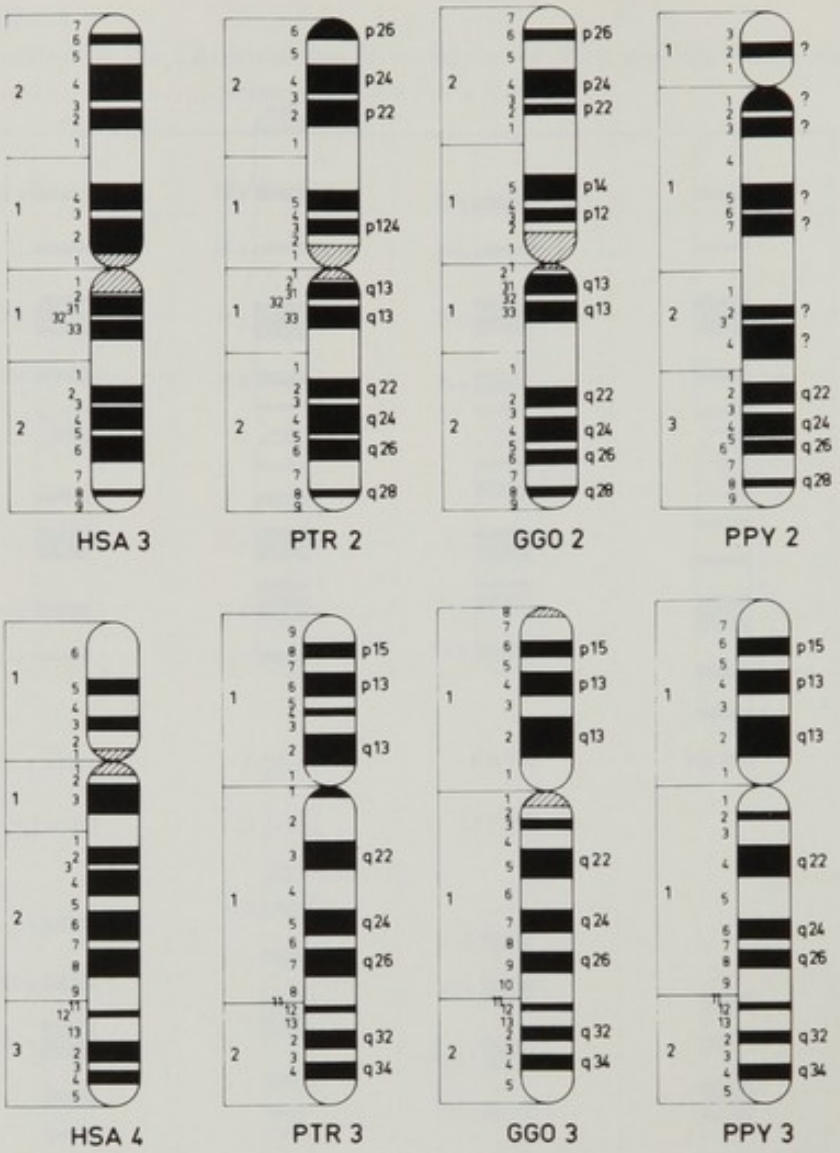

Fig. 7. The band diagrams for human chromosomes 3 and 4 inclusive and their supposed homologues in the great apes. Published with kind permission of the National Foundation.

(Dutrillaux, 1975; Miller *et al.*, 1974) using R- and G-banding techniques respectively, raising the question of why various genera within the same family and superfamily should show such dissimilarity to one another. The dilemma has been compounded by studies on cercophithecoid species, principally baboons, rhesus and African green monkeys (Stock and Hsu, 1973; Seth *et al.*, 1976), in which some chromosomes clearly appear to fit the human pattern far better than do those of gibbons and siamangs.

In particular, the studies of Stock and Hsu (1973) indicate that regions of chromosomes corresponding to parts of human chromosomes, including 1, 5, and 12, have been retained more or less intact, suggesting an ultraconservation of particular genetic sequences. The same authors have also shown that the karyotype of the African green monkey differs from that of the rhesus monkey through a series of rearrangements, mainly translocations.

IV. CHROMOSOME POLYMORPHISM

Inherited variation in the size or staining intensity of particular bands adjacent to the centromere has been described in human chromosomes 1, 3, 4, 9, 13, 14, 15, 16, 21, 22, and Y, at various frequencies. The variant band has in all instances been found to consist of constitutive heterochromatin which could either by demonstrated by quinacrine staining or C band staining variations (Pearson *et al.*, 1973) such as the Giemsa-11 technique.

Early fluorescent studies (Pearson *et al.*, 1971) noted the presence of intensely fluorescent satellites on the acrocentric chromosomes of the chimpanzee and gorilla which are apparent at a higher frequency than found in man (7–9 satellited chromosomes per animal in the 7 chimpanzees examined, Pearson *et al.*, 1973). Curiously, fluorescent satellites have never been observed by the author on chimpanzee chromosome 16 (HSA 15), a point also noted by Bobrow and Madan (1973) (Fig. 8). Lin *et al.* (1973), while investigating a population of 22 chimpanzees, claimed that the satellite fluorescence was variably present on all acrocentric chromosomes. However, examination of their karyotype photographs suggest they have placed chimpanzee chromosome 17 (HSA 18) in the 16 position (HSA 15), and as a result PTR 16 may indeed have no fluorescent satel-

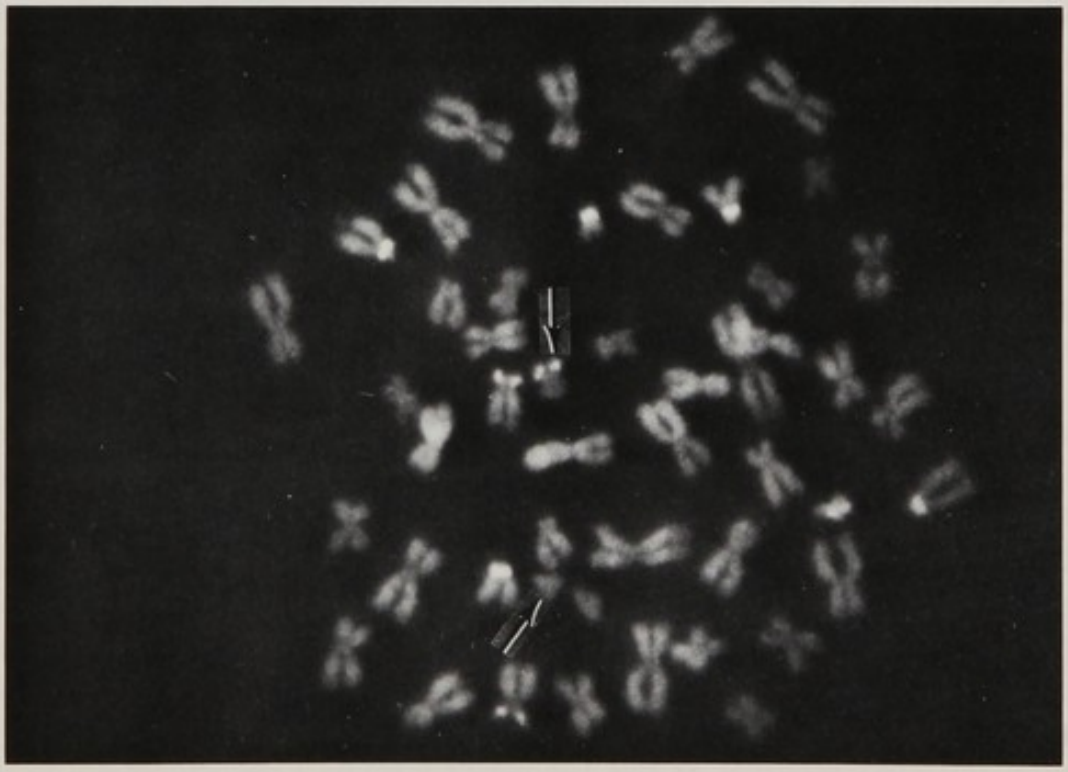

Fig. 8. Quinacrine stained *Pan paniscus* chromosomes. Arrows indicate the No. 23 chromosomes (HSA 22) which in this animal appears to be polymorphic.

lites. Bobrow and Madan (1973) suggested that the lack of fluorescent satellites on this chromosome may in some way be related to the presence of Giemsa-11 stained heterochromatin in the proximal part of the long arm and not the short arm.

The long ago noted karyological difference between *Pan troglodytes* and the pigmy chimpanzee, *Pan paniscus,* of a pair of small acrocentrics in the former being replaced by a metacentric chromosome in the latter (Hamerton *et al.*, 1963) has been ascribed to the presence of extremely large fluorescent short arms and satellites on the *P. paniscus* chromosome 23 (HSA 22) (Pearson, 1973; Lejeune *et al.*, 1973). We recently examined another specimen and found that only one *P. paniscus* chromosome 23 had fluorescent satellites (Fig. 8), indicating that this was either a polymorphic difference within the species or that the animal examined was a hybrid between *P. troglodytes* and *P. paniscus*. There was no evidence to suggest the latter.

A type of polymorphism is present in the chimpanzee and gorilla which has not yet been found in man, namely, the presence of variable Q bands close to the telomeres of both of the long and short arm of the smaller chromosomes (Fig. 9). These distal polymorphisms are extremely variable and can most clearly be noted on chimpanzee chromosomes 20, 21, 22, and 23 (HSA 19, 20, 21, and 22, respectively). Miller *et al.* (1974), Dutrillaux *et al.* (1973), and Pearson (1973) noted both an extensive telomeric and satellite polymorphism in the gorilla also (Fig. 9) which can be identified by either C- or Q-banding techniques. When C-banding is used, terminal heterochromatic bands can be found on most of the gorilla chromosomes (Fig. 10). To what extent these are all polymorphic is not clear at present. Two principal differences between the polymorphisms appear to be present in man and the chimpanzee and gorilla. First, the secondary constriction variations found in chromosome 1 in man are absent in the great apes. Indeed a comparable band to 1q12 in man is either entirely absent or extremely reduced in these species. Jones (1976) has speculated that the absence of this band may be associated with an apparent absence of satellites I and II in the great apes and that the origin of these satellites has played an important role in human evolution (see Chapter 9). Second, Giemsa-11 staining chromatin, which appears to correspond to the distribution of human satellite III (Jones, 1973), is present in larger quantities in chimpanzee and gorilla than in man, and perhaps in more or less the same quantity in man and orangutan (P. L. Pearson, personal observations) (Figs. 11, 12, 13, and 14). Jones (1976) has demonstrated that a DNA corresponding to human satellite III is apparently absent in all primates except the great apes, and our initial observations with the distribution of Giemsa-11 heterochromatin in the karyotype of the rhesus monkey tend to confirm this. The variation of this Giemsa-11 staining heterochromatin in the great apes has not yet been adequately documented to show whether it is always

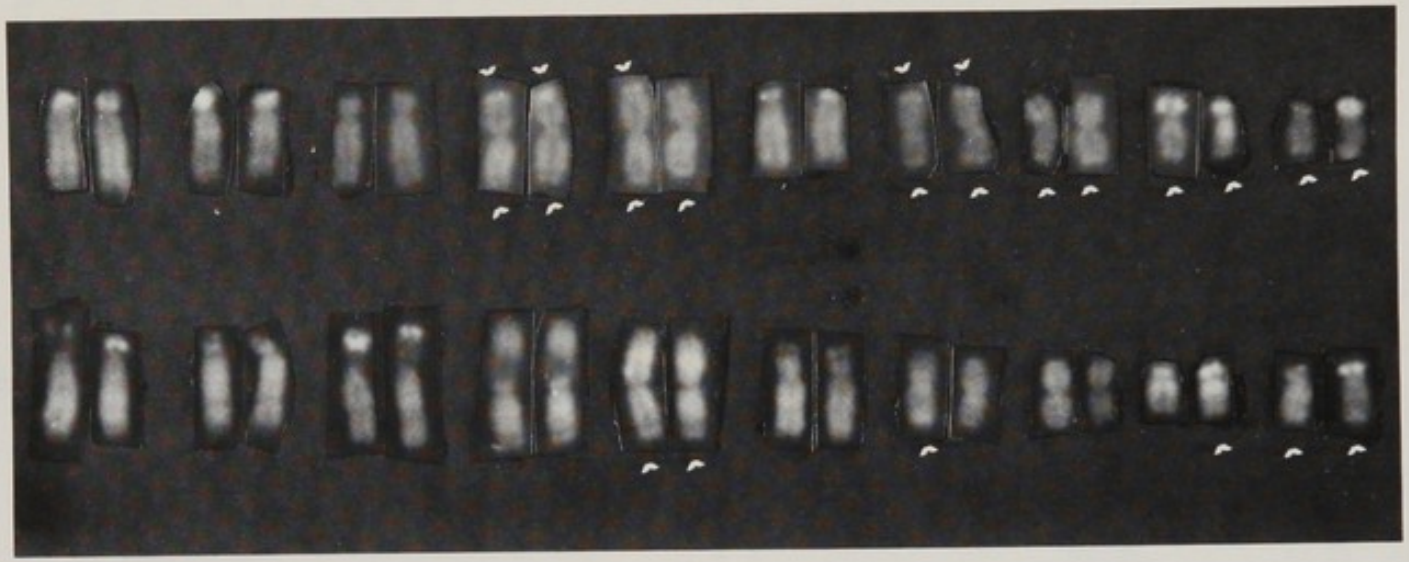

Fig. 9. Fluorescent polymorphisms in chimpanzee chromosomes 14 to 23 (above) and gorilla chromosomes 14 to 23 (below). The chromosomes have been arranged to correspond to the human chromosome sequence 13 to 22. Telomere polymorphisms are indicated by arrows. Variably brilliant fluorescent satellites or short arms are also present on most of the acrocentric chromosomes.

Fig. 10. C-banding in the gorilla. The majority of telomeres and centromeres appear to be C-band positive. Compare this pattern to that shown in Figs. 12 and 13, where only C-banding corresponding to "satellite III" chromatin is present.

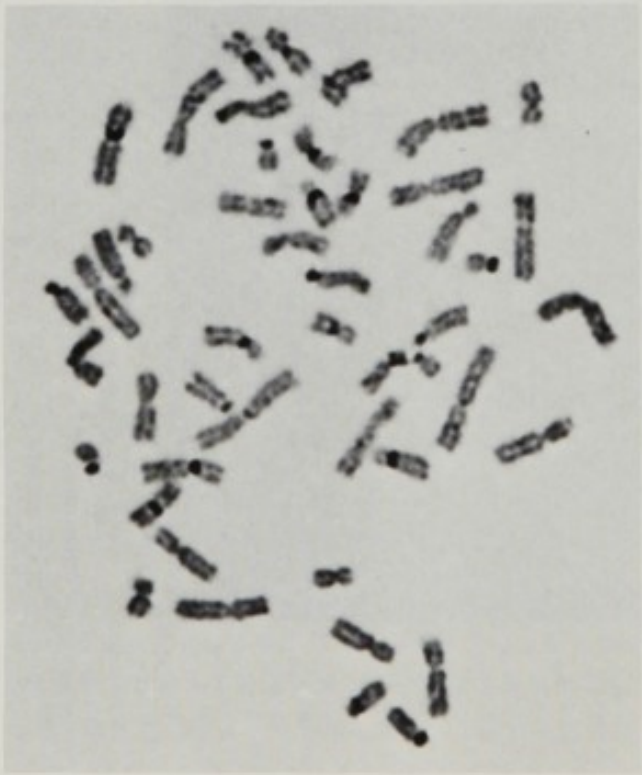

Fig. 11. Giemsa-11 staining in the chimpanzee.

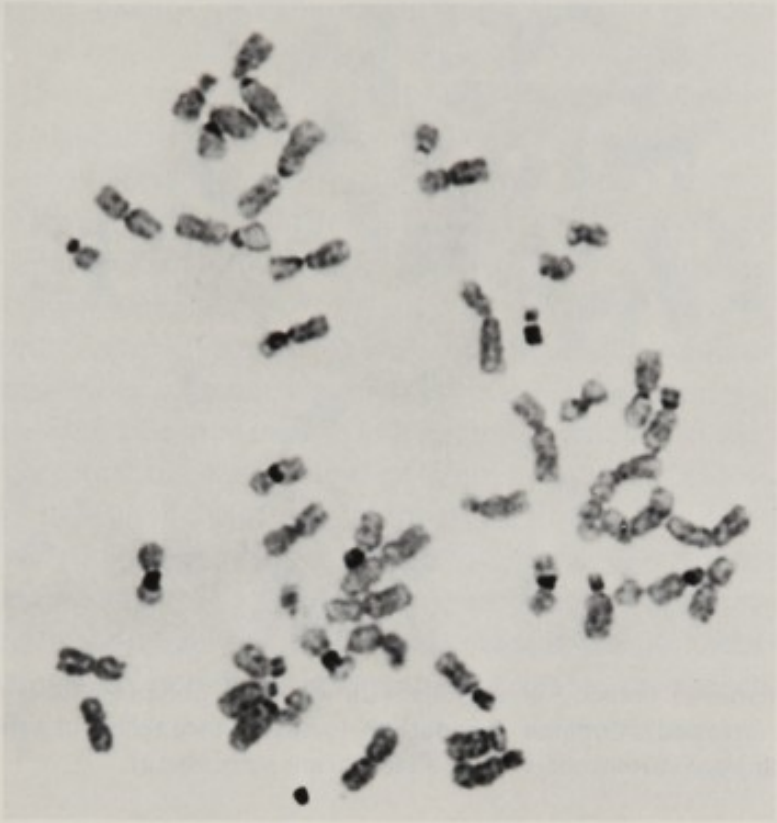

Fig. 12. Giemsa-11 staining in the gorilla.

polymorphic. Present indications are, however, that heterochromatin located on the short arms and/or satellites of acrocentric chromosomes and the telomeres of other chromosomes can clearly vary, but insufficient information is available to know whether heterochromatin located next to the centromere also varies. In man, little or no variation of this type of centromere-located heterochromatin has been seen apart from the bands on 1, 9, and 16 and to a lesser extent 19. The fluorescent bands frequently seen at the centromere of chromosomes 3 and 4 do not appear to vary with the Giemsa-11 technique and are, indeed, very reduced in size. It is of interest to note that the gorilla chromosome 3 (HSA 4) has an intensely fluorescing band next to the centromere of the type occasionally seen in some human chromosome 4's (Pearson *et al.*, 1973), and this has been noted to be polymorphic in some animals (Pearson, 1973; Miller *et al.*, 1974).

It must be realized that only limited numbers of animals have been investigated and that our estimates of species variability are still speculative. It should also be noted that, despite the limited numbers examined, gorillas have been discovered to possess an extra small chromosome that is not associated

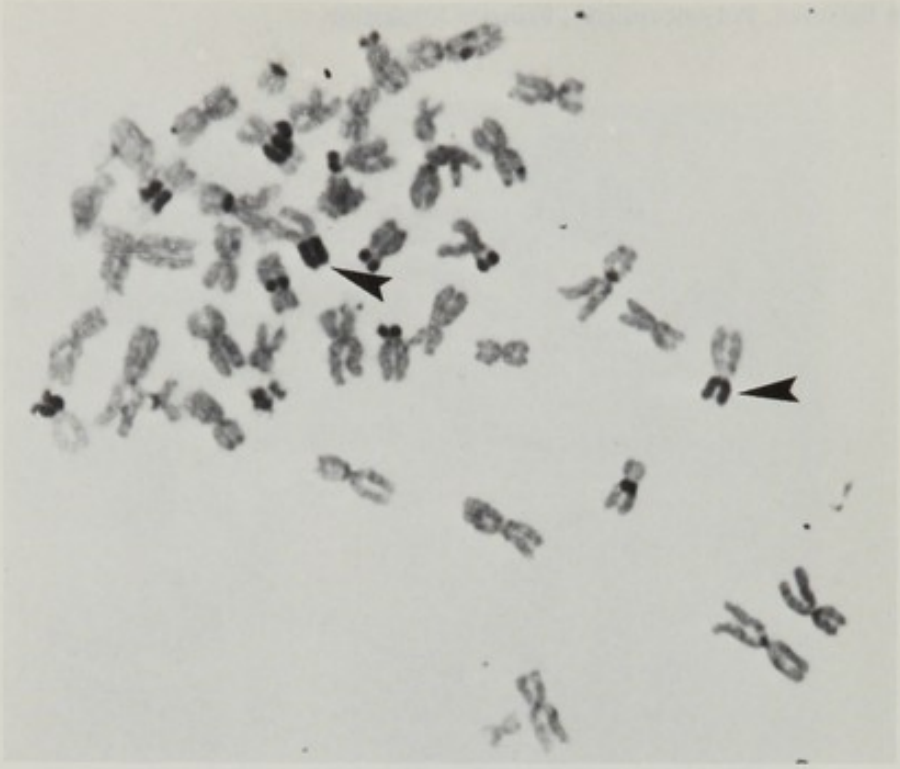

Fig. 13. Giemsa-11 staining in a gorilla with extensive polymorphisms on chromosome 15 (HSA 15) (arrowed). Compare this pattern to that shown in Fig. 12 prepared from an animal in which large chromosome 15 polymorphisms were absent.

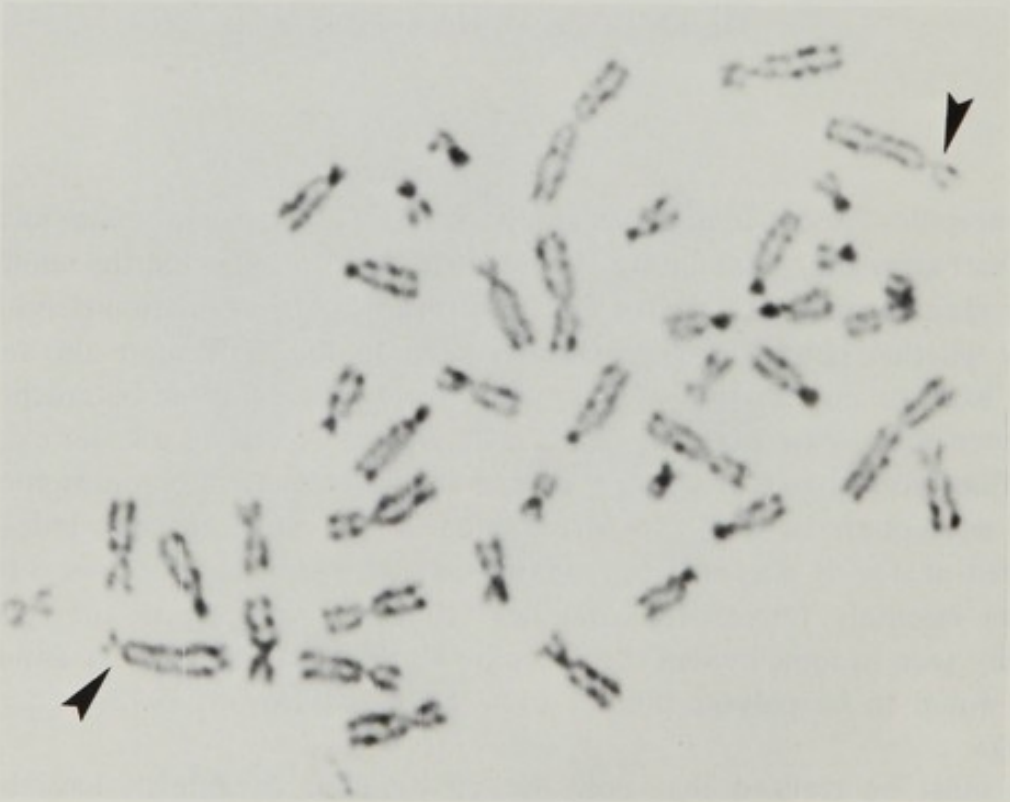

Fig. 14. Giemsa-11 staining in the orangutan. Arrows indicate a possible pericentric inversion polymophism on chromosome 2 (HSA 3).

with any apparent phenotypic abnormality (de Grouchy *et al.*, 1973; J. de Grouchy, personal communication) of the type occasionally found in human families, and is thought to be an "accessory" chromosome. Pericentric inversions have also been described in chromosome 2 of the orangutan (Turleau *et al.*, 1975; P. L. Pearson, personal observations) (Fig. 14) and in one of the chromosomes in the gibbon (Tantravahi *et al.*, 1975), suggesting a comparatively high frequency of this type of structural rearrangements, considering the limited number of specimens examined.

Using antibodies specific for the DNA base 5-methylcytosine, Schnedl *et al.* (1975) demonstrated large quantities of this base in heterochromatic regions in the gorilla, somewhat less in man, and only a limited amount in the chimpanzee. The authors point out that the amount of 5-methylcytosine chromatin appears to vary within and among individuals, thus constituting yet another form of polymorphic variation, and that it is almost certainly associated with the evolution of hominoid satellite DNA's. They further conclude that the situation in the chimpanzee is probably a result of a decrease in the number of repetitious sequences. There is no published information available on the distribution of 5-methylcytosine in other primates.

V. DISTRIBUTION OF rDNA IN PRIMATES

With the use of *in situ* hybridization, Henderson *et al.* (1972) demonstrated that the loci coding for the 18 and 28 S ribosomal DNA cistrons were present in the short arms of all acrocentric chromosomes in man. The same group (Henderson *et al.*, 1974b) subsequently showed the presence of the same loci in the short arms of the chimpanzee acrocentric chromosome 14 (HSA 13), 15 (HSA 14), 22 (HSA 21), and 23 (HSA 22) but failed to detect them in chromosome 16 (HSA 15), supporting the conclusion derived from fluorescence and heterochromatin distribution studies that this chromosome in the chimpanzee differs from the other acrocentrics. Information on the situation in the gorilla and orangutan is lacking at present, but it can be predicted that the short arms of the acrocentric chromosomes will be shown to contain RNA cistrons as in man and the chimpanzee. It is of interest that the two chimpanzee chromosomes 12 and 13, which are believed to comprise chromosome 2 of man, were not found to carry rDNA cistrons. Studies carried out on the rhesus monkey (Henderson *et al.*, 1974a) demonstrated the presence of a single metacentric chromosome containing rDNA, suggesting that the distribution of ribosomal cistrons has varied considerably in the evolution of primate karyotypes.

The 5 S rDNA cistrons are carried in the distal half of 1q in man (Steffensen *et al.*, 1974), and this observation has recently been confirmed in chromosome 1 of the great apes (Warburton *et al.*, 1976; see also Chapter 3).

VI. GENE MAPPING STUDIES

During the last few years, enormous strides have been made in human gene mapping studies, principally through the use of somatic cell hybrid analysis (see, for example, Rotterdam Conference, 1974). Approximately 100 genes have now been assigned to individual human chromosomes, and in some instances the actual location of individual genes has been mapped within the chromosome in relation to the position of bands. In the preceding sections, it was noted that some of the chromosomes present in the primates were identical to those in man in their banding pattern while others showed greater or lesser differences. It is of fundamental interest to know whether chromosomes defined as homologous on the basis of banding patterns (see Table I) carry identical gene loci and also whether in situations in which the banding pattern relationships are not so clear, such as between HSA 14 and GGO 13, the presence or absence of gene loci can be used to clarify their phylogenetic relationships. Accordingly, during the past 2 years, we have been engaged in an extensive cell hybridization program using cells of various primate species and the Chinese hamster. This work is being done in collaboration with Dr. Meera Khan in Leiden and Dr. A. Westerveld in Rotterdam (Pearson *et al.*, 1976).

A total of 12 primary clones and 18 subclones have been analyzed both for isoenzyme and chromosome content from the gorilla and 10 primary clones from the chimpanzee. The results are shown in Table II (see also Warburton and Pearson, 1976). Chromosome analysis was performed using a variety of banding techniques on each clone and included quinacrine fluorescence, Giemsa-trypsin banding (Fig. 15), and Giemsa-11 staining (Bobrow and Cross, 1974). We find that the latter technique is a powerful method for estimating the total number of primate chromosomes present and for making a provisional identification of particular chromosomes (Fig. 16), which can then be confirmed by other banding techniques. The following general and specific conclusions can be drawn from the existing data:

1. In general those chromosomes which are identical to human chromosomes have also been found to contain the same enzyme loci. Thus the homologues of human chromosomes 1, 4, 5, 10, 11, 12, 17, 18, 21, and X appear to carry the same enzymes as in man.

2. One exception to this is the homologue of chromosome 6 which in both the gorilla and chimpanzee does not segregate with SOD_2, and in the case of the gorilla does not with PGM_3. This in itself is an extremely important observation since chromosome 6 in man is known to carry the HL-A loci, and this will have to be confirmed using other new markers such as GLO.

3. In the gorilla the isoenzyme NP, which in man is linked to chromosome 14, segregates with chromosome 13. Chromosome 13 of the gorilla was thought to be homologous to HSA 9 on the basis of banding patterns by Dutrillaux *et al.*

TABLE II
Comparative Gene Mapping of Man, Chimpanzee, and Gorilla[a]

Man		Chimpanzee		Gorilla	
Chromosome	Enzyme	Homologous chromosome	Enzyme present	Homologous chromosome	Enzyme present
1		PTR 1		GGO 1	
	PPH		+		+
	PGD		+		+
	PGM-1		+		+
	UGP		?		?
	FH		?		?
	GUK				+
	Pep-C				+
2p		PTR 12		GGO 12	
	MDH-1		+		+
4		PTR 3		GGO 3	
	PGM-2		+		+
5		PTR 4		GGO 4	
	Hex-B		?		+
6		PTR 5		GGO 5	
	SOD-2		–		–
	PGM-3				–
7		PTR 6		GGO 6	
	B-GUR		+		
9		PTR 11		?	
	AK-1		+		
11		PTR 9		GGO 9	
	LDH-A		+		+
12		PTR 10		GGO 10	
	LDH-B		+		+
	Pep-B		+		+
	TPI		+		+
14		PTR 15		?GGO 13, ?GGO 18	+
	NP		+		+
15		PTR 16		GGO 15	
	MPI				+
	Hex-A		?		+
	PK-3		?		+
17		PTR 17		GGO 19	
	TK		?		+
18					
	Pep-A		?		+
19					
	GPI				+
20		PTR 21		GGO 21	
	ITP		+	ITP associates with NP (Meera Khar, *et al.*, 1976)	?
21		PTR 22			
	SOD-1		+		+
X		X		X	
	HPRT		+		
	G6PD		+		+
	PGK		+		+
	a-GAL		+		+

[a]Full names of the enzyme abbreviations are to be found in Rotterdam Conference (1974).

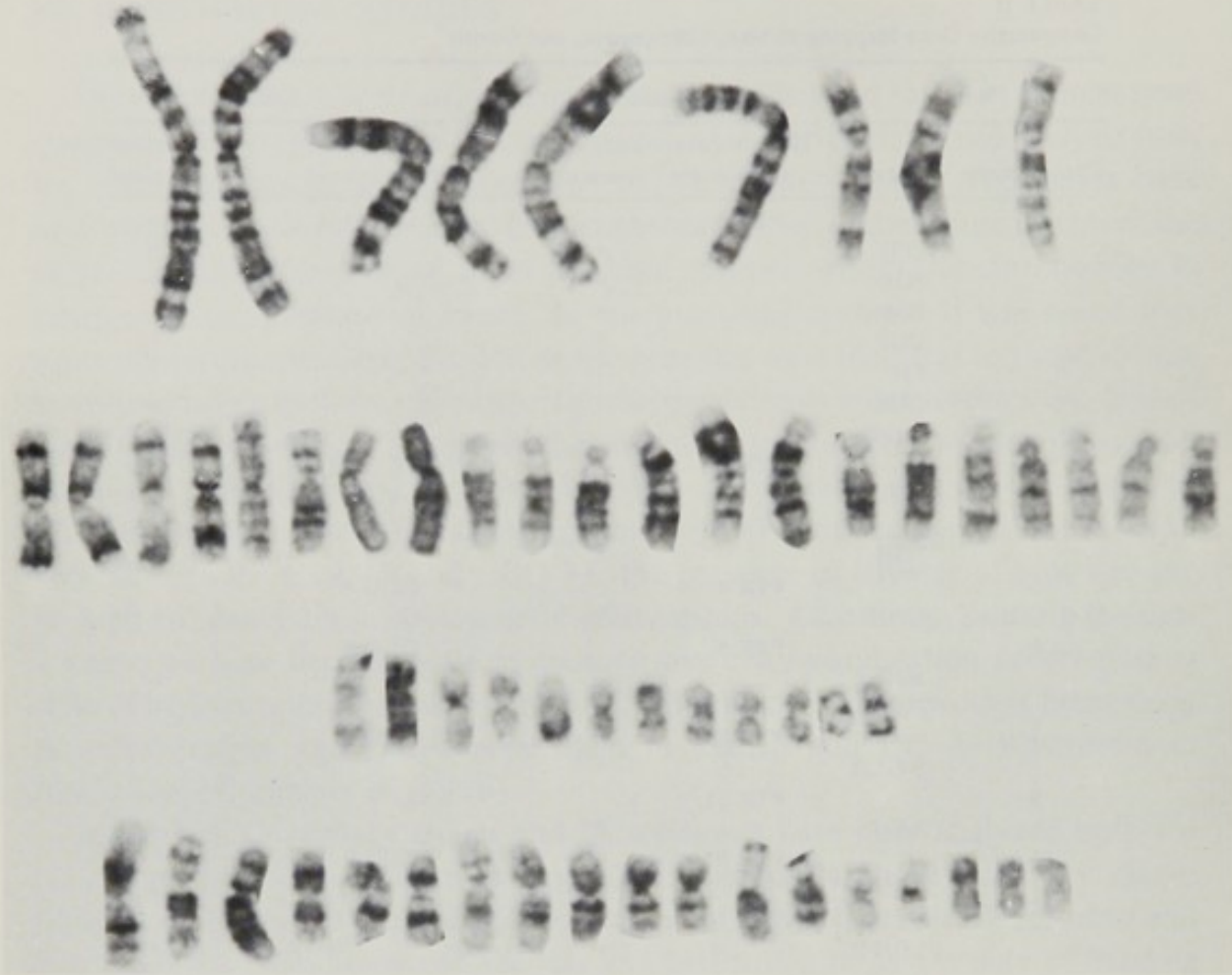

Fig. 15. Giemsa-banded karyotype of a gorilla–Chinese hamster hybrid cell. The gorilla chromosomes are arranged on the bottom row.

(1975) but the isoenzyme segregation indicates that a more probable gorilla homology for this is chromosome 18. We have also shown that ITP is syntenic (Meera Khan *et al.*, 1976) with NP in the gorilla, but in man ITP appears to be carried on chromosome 20 indicating undetected structural differences for these chromosomes.

4. We have been unable to demonstrate the presence of two enzymes, namely UGPP and FH in either the chimpanzee or gorilla hybrids. These two enzymes which are present on chromosome 1 in man are thus either not expressed in the cell hybrids or are carried on another primate chromosome which has been lost.

5. We have shown that the enzymes MPI and PK_3 which are syntenic as in man, are present on gorilla chromosome 15 (HSA 15), but have failed to demonstrate that they are also carried on the chimpanzee chromosome 16 (HSA 15), indicating another curious difference of this chromosome.

6. There appears to be a clear relationship in both the gorilla and chimpanzee that MDH-1 segregates with the respective homologues of 2p in man, confirming

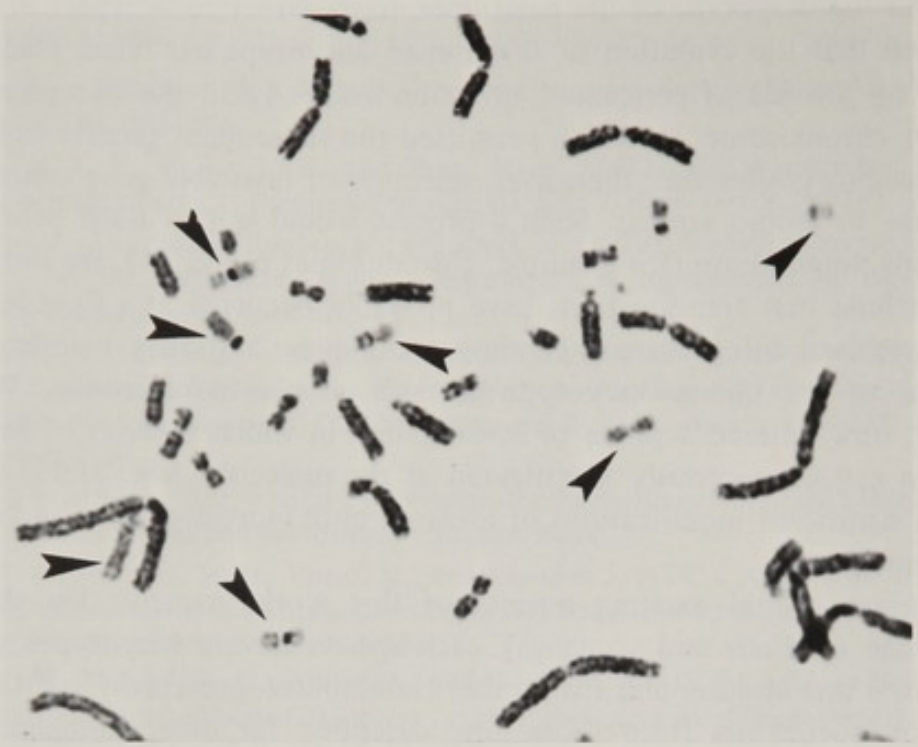

Fig. 16. Giemsa-11 stained chimpanzee–Chinese hamster hybrid. The more lightly stained chromosomes (arrowed) belong to the chimpanzee. The presence of a deeply staining band on a chimpanzee chromosome can frequently permit its specific identification. In this cell, for example, there are two No. 11 (HSA 9) chromosomes with a band in the long arm adjacent to the centrally positioned centromere.

the proposed origin of the human chromosome 2 short arm. There is as yet no hybrid cell evidence to confirm the long arm origin of human chromosome 2.

The results in the chimpanzee have been confirmed and augmented by other groups: Finaz *et al.* (1975), Chen *et al.* (1976) and Orkwiszewski *et al.* (1976) (see Warburton and Pearson, 1976). The latter two groups have demonstrated the localization of the TK and galactokinase genes to chimpanzee chromosome 19 (HSA 17), which is believed to only differ from the human 17 by one pericentric inversion (Bobrow and Madan, 1973; Turleau and de Grouchy, 1973).

VII. ARE BANDING PATTERNS A TRUE REFLECTION OF GENETIC HOMOLOGY?

In the previous section it was shown that in general, a similarity in banding pattern is associated with a similar gene content, at least for the limited number of isoenzyme markers studied. Dutrillaux (1975) attempted to demonstrate that practically every band, with the exception of constitutive heterochromatin

present in the karyotype of the great apes, has a direct counterpart in man. He concluded that the evolution of the human karyotype has taken place almost entirely by a series of pericentric inversion events (with the exception of the origin of chromosome 2), which permitted the subsequent genetic isolation of small breeding groups and, thereafter, selection of favorable gene combinations giving rise to *Homo sapiens*. Such a process would not in itself produce the exceptions noted above (for example, chromosomes 6 and 14). We must therefore conclude that translocations have probably occurred at a finer level than can be resolved using current banding techniques, and that theories on the evolution of the human karyotype are still an oversimplification. We have, however, now entered a phase of investigation in which theories of karyotype evolution can be rigorously investigated at the molecular level and is likely to result in significant modifications of ideas on both individual band and chromosome homology.

There are several exciting aspects of this work, namely: Do the Symphalangidae (gibbons and siamangs), each species having karyotypes very different from one another and from other Hominoidea, possess different syntenic groups of isoenzymes from those now described for man, chimpanzee, and gorilla? Do the Old World monkeys represented by the macaques, although taxonomically further removed from man than the Symphalangidae, have syntenic groups more similar to the human pattern? Although it is my prediction that this will turn out to be the case, the information gained will, in any event, give us greater insight into the relationship between gene and chromosome band distribution as markers of evolution in general and, in particular, of man.

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9

Repetitive DNA and Primate Evolution

K. W. JONES

I. INTRODUCTION

The problem of how species evolve is far from a solution. From what is known about protein evolution, it does not appear that mutations in structural gene sequences can completely explain the differences between closely related species such as man and chimpanzee (King and Wilson, 1975) and thus we must look elsewhere for other significant forces of the evolutionary process.

A new initiative with respect to this problem has been provided following the discovery of the repeated nature of nucleotide sequences in eukaryotic genomes

(Waring and Britten, 1966). Such sequences are evolving with impressive rapidity. The significance of this is obscure since much repetitive DNA is defined kinetically and is of unknown function. The most highly repeated and fastest evolving DNA's, satellite DNA's, however are better characterized because they can be isolated and easily purified. They are concentrated mainly in constitutively heterochromatic regions of chromosomes. In this chapter a sketch is given of the evolution and structure of repeated DNA in primates, with emphasis on the satellite DNA's of man and the higher primates. The possible role of these DNA fractions in evolution is discussed with reference to examples derived from nonprimate sources. This role is seen essentially as one of influencing the evolution of linkage groups through possible effects on crossing-over and of influencing karyotypic evolution through centric fusion.

II. REPEATED AND NONREPEATED DNA

Because of the problems of identifying and purifying homogeneous classes of repetitive DNA, their evolution and arrangement has largely been studied by kinetic methods, except in the case of satellite DNA's which can be purified in the native state.

The fraction of DNA detected as repeated or as nonrepeated heavily depends on the conditions under which the analysis is performed. Repeated DNA is kinetically defined as that fraction of DNA which reassociates under conditions of monovalent cation concentration and time which would not permit any reassociation of nonrepeated sequences. Under stringent conditions, which require nearly perfect base pairing in order for duplexes to become established, only well-matched repeated sequences will reassociate. Other sequences will be classified as nonrepeated. Lowering the criteria (usually by lowering the temperature) allows less well-matched duplexes to form and thus includes more DNA within the classification of repeated. This means that the kinetic definition of a repeated sequence in practice is necessarily empirical because the degree of exactness of repeats in different classes of repeated DNA is variable, and conditions can be devised under which few sequences will be seen as repeated. The analysis of genomal DNA is usually conducted under conditions of salt concentration and temperature which allow the reassociation of most or all of the DNA and which measure this as a function of the product of the initial DNA concentration in moles per liter and the time of incubation in seconds (Cot; Britten and Kohne, 1968) to permit comparison between DNA's with extremely different rates of reassociation. To accommodate the wide limits shown by complex genomes, the values obtained are plotted on a logarithmic scale.

The frequency of repetition of a given nucleotide sequence family determines its reassociation rate under given conditions. Relatively highly repeated nucleo-

tide sequences reassociate rapidly, less repeated sequences more slowly, while nonrepeated sequences reassociate slowest of all. If a genome contains a relatively high proportion of repeated nucleotide sequences, its DNA will approach the point at which 50% has reassociated faster than a similar sized genome containing a relatively low proportion of repeated sequences. This will reflect in the shape of the Cot curve. Comparing the Cot curves of related DNA species will therefore show how genomes have altered relatively during evolution, how undiverged DNA has been added to genomes and on the assumption that genomes have maintained approximately similar sizes, how the proportions of repeated and less repeated DNA have altered.

III. COMPARATIVE ANALYSIS OF PRIMATE REPEATED DNA

With reference mainly to two well-defined primate groups, the superfamilies Cercopithecoidae and Anthropoidae, Gummerson (1972) showed that quite large alterations have occurred in the composition of the repeated genome within the same superfamily in the primates. In the Cercopithecoidae, baboon (*Papio cynocephalus*) and African green monkey (*Cercopithecus aethiops*) have about 50% more repeated DNA than the rhesus monkey (*Macaca mulatta*) (Fig. 1,A–C). Such differences probably have occurred within the past 15 million years by the addition of new DNA, since the DNA involved is relatively undiverged. The higher primates, represented by the gibbon, chimpanzee and human, have undergone relatively much less alteration in genomal composition.

All of the primates studied contain a considerable amount of DNA with a repetition frequency greater than 10,000 (Cot 0.1). This ranges from almost 35% in the African green monkey and the baboon to about 14% in the rhesus monkey. Some of this DNA is satellite DNA. The genome of African green monkey, for example, contains one satellite DNA (1.699 gm/cm^3) comprising about 20% of the total (Maio, 1971). It also contains another satellite (1.711 gm/cm^3) which appears to vary considerably in amount (Kurnit and Maio, 1974) which may comprise 11% of the total DNA. Baboon similarly has a 5–10% satellite, with a buoyant density (1.700 gm/cm^3) similar to that of African green monkey, and a heavy satellite which has not been characterized (Prosser, 1974). The light satellites in both are also similar in their denatured density (1.718 and 1.714 for African green monkey and baboon, respectively) and in their reassociation rate which resembles that of mouse satellite. Baboon satellite is about as diverged as mouse satellite, having a difference of 4.5°C between its native and renatured melting points (ΔT_m; see Section IV) suggesting a fairly recent origin. It has been shown to be localized in centromeric heterochromatin on the majority of the chromosomes (Prosser, 1974).

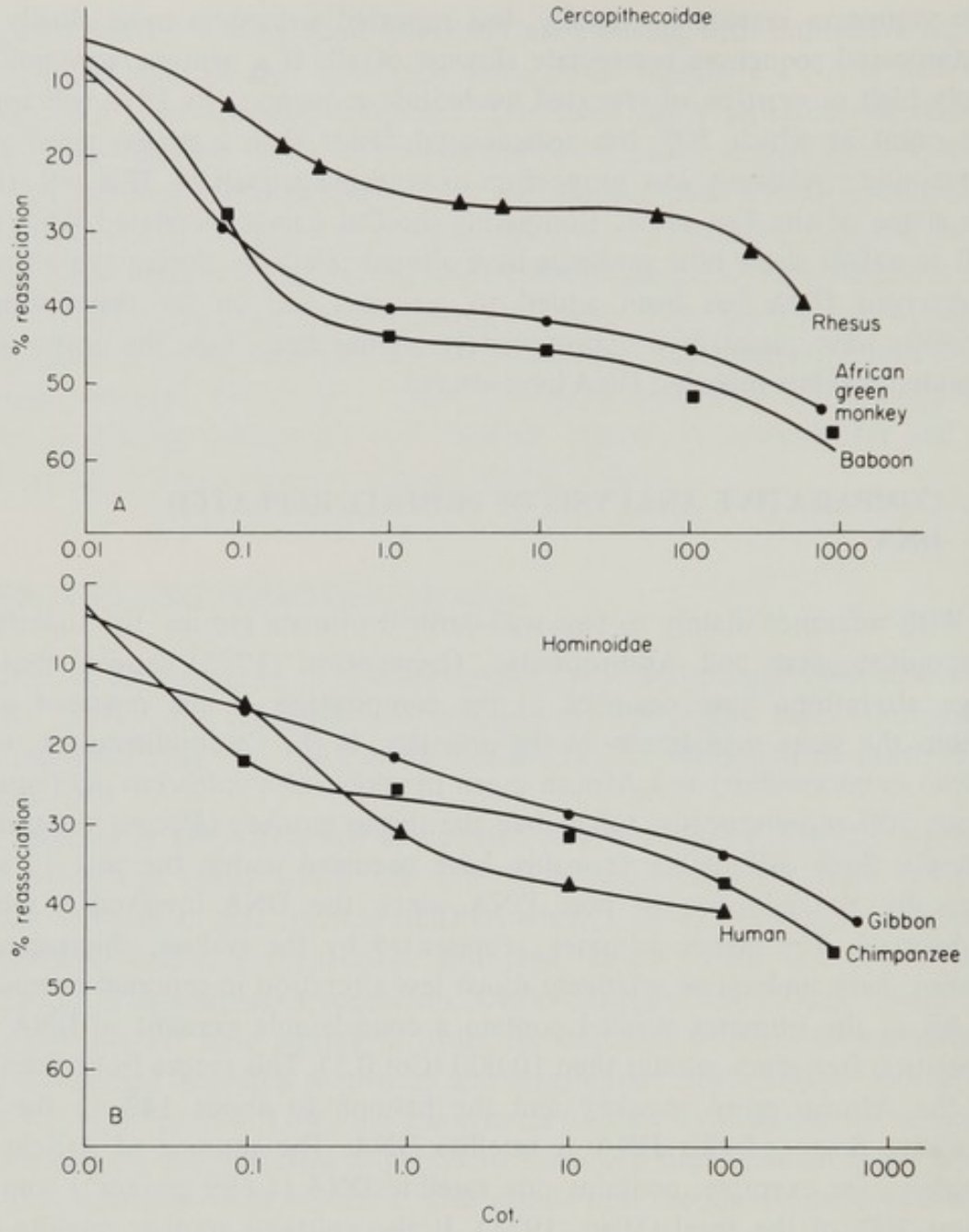


Fig. 1A, B, and C. Cot curves of the DNA isolated from representatives of the order Primates. DNA was reassociated in 0.12 *M* phosphate buffer at 60°C and fractionated on hydroxyapatite. (Adapted from Gummerson, 1972.)

A large proportion of the difference in Cot distribution of primate genomes is thus attributable to satellite DNA evolution. However, there are also other repeated DNA differences.

The amount of DNA with a low frequency of repetition (Cot 1 to Cot 100) containing sequence families of from 1000 to 10 members is different in the Hominoidae, Cercopithecoidae and Lorisoidae. The Hominoidae have roughly one third of their repeated sequences in this range, while the Cercopithecoidae

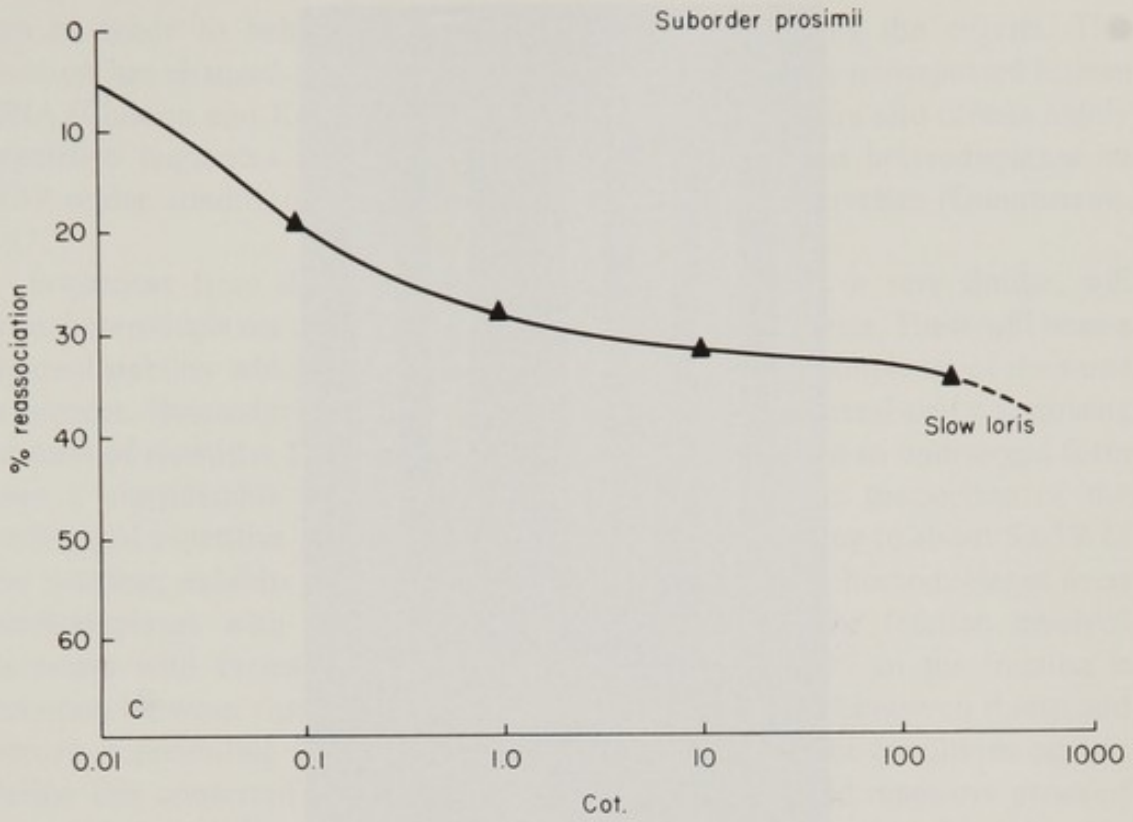


Fig. 1. *(continued)*

have most of their repeated DNA in copies of more than 1000. The Lorisoidae also contain such sequence families but additionally show a few families reassociating between Cot 1 and Cot 10. These orders show few families in the range of Cot 10 to Cot 100.

The prominent satellite DNA's in members of the Cercopithecoidae are clearly revealed by the use of restriction enzymes (Fig. 2). These are nucleotide sequence-specific endonucleases from microorganisms which cleave double-stranded DNA within a short defined sequence of about half a dozen nucleotide pairs (Kelly and Smith, 1970). Repeated DNA containing a cleavage site yields a class of similar sized fragments, or multimers of a basic fragment if there has been modification of the restriction site due to mutation or other processes. Fractionation of such restricted DNA on agarose gels produces a banded pattern which reflects the molecular weight classes of the fragments. Satellite DNA's in many groups of organisms exhibit such patterns (e.g., Southern and Roizes, 1973; Botchan *et al.*, 1973), but at this time not much advantage has been taken of this approach to the study of primate repetitive DNA.

The impression created by kinetic and restriction analysis of the repetitive genomes of primates is one of considerable evolutionary dynamism. In spite of this, there is evidence of the conservation of certain types of repetitive DNA which includes sequences of known, as well as of unknown, function.

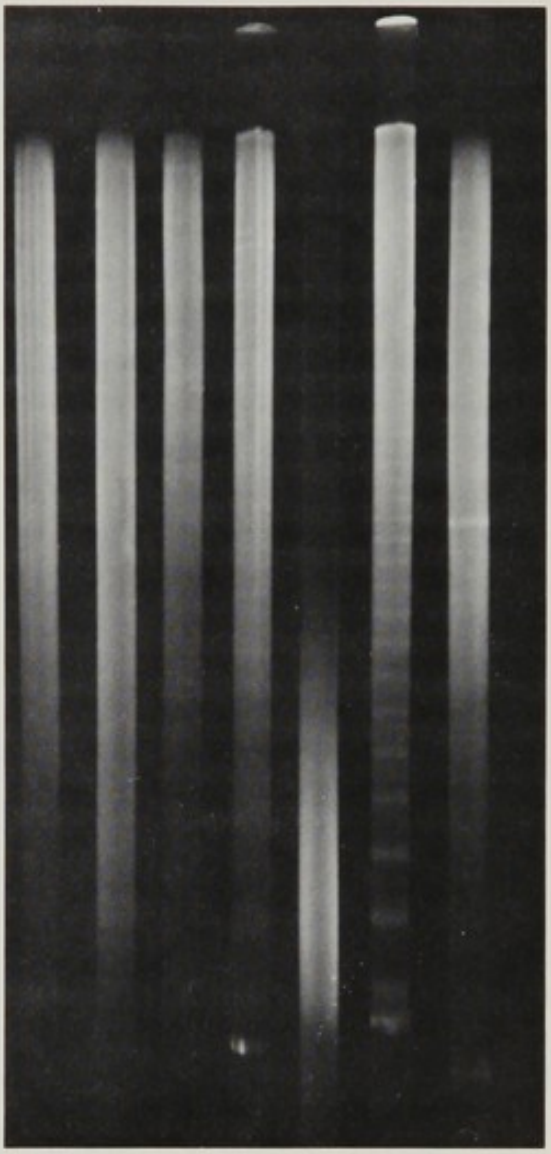


Fig. 2. Repetitive DNA in evolution as shown by DNA digestion with EcoRl restriction endonuclease digests of a range of primate species separated for comparison on 1% agarose gels. The species DNA shown are, left to right, gibbon, marmoset, baboon, macaque, orangutan, vervet monkey, and *Loris*. Prominent bands are visible in the DNA of the *Loris*, vervet, and macaque. (Those in the latter DNA, however, were difficult to photograph.) Marked differences are thus revealed in the species distribution of highly repeated DNA containing the EcoRl restriction sequence (see text).

IV. CONSERVATION OF REPETITIVE DNA SEQUENCES IN PRIMATES

It is usually supposed that repetitive sequences alter more rapidly in evolution than single copy DNA since the former are, in general, thought to have less precisely sequence-dependent functions. However a large fraction (30%) of human repeated DNA which reassociates with gibbon DNA under low criteria

can be made to behave like nonrepeated DNA by raising the criteria. This fraction has changed at a rate not greatly different from the nonrepeated human DNA (Chiscon and Kohne, cited in Kohne, 1970). There are also certain highly repetitive sequences in primates which may be isolated as heteroduplexes on HAP under conditions which show them to be very conservative (Gummerson, 1972).

Sequences from different species which are identical, or very similar, will form heteroduplexes upon being renatured under strict criteria. These will have a thermal stability which is very close to that shown by homoduplexes of the same sequences. Heteroduplex studies among primate genomes reveal that a surprising amount of repetitive DNA appears to have been conserved in an undiverged form over a considerable period of evolutionary time. Thus a proportion of the undiverged repetitive DNA of the rhesus monkey, amounting to about 5.65% of the genome, exhibits the same thermal stability either as homoduplexes or as heteroduplexes with baboon and with human DNA. The fraction involved decreases with increasing evolutionary distance. Thus 80% of the fraction is common between rhesus and baboon as compared with 50% between rhesus and human, representing 3.45% of the human genome. Under the conditions used to define this conserved fraction, it would not have contained repetitive genes of known function. Its significance is unknown but highly repetitive DNA forming part of the sex chromosomes in certain orders is also conservative, as discussed later.

Conservation of the intermediately repetitive genes of known function, such as for example, the ribosomal DNA structural sequences, is well known (Brown *et al.*, 1967). Ribosomal DNA from insects, for example, will form heteroduplexes with amphibian rRNA (Pardue *et al.*, 1970) and amphibian rRNA will form heteroduplexes with human ribosomal cistrons (Evans *et al.*, 1974). The quality of the heteroduplexes formed using ribosomal RNA shows a gradual reduction over a range of species from bacteria to higher organisms (Bendich and McCarthy, 1970) and there has been a steady evolution of such genes involving an increase in the (G + C/A + U) ratio from fungi to flowering plants (Amaldi, 1969).

The relationship between the thermal stability of a reassociated DNA and the extent of nucleotide mismatching involved in reassociation has been very thoroughly studied (e.g., McCarthy and Farquhar, 1972). A value of 1°C reduction in T_m (ΔT_m) of the reassociated DNA compared with the native DNA is taken to indicate mismatch of 1.5% of nucleotide pairs. Within the primates, 28 S rDNA divergence has occurred to a measurable extent in the few species where comparisons are available (Table I). The reduction in the thermal stabilities of heteroduplexes reflects reasonably accurately the extent of species divergence as determined palaeontologically, and when adjusted for years of evolutionary divergence, yield a roughly constant value. Calculations based upon this data

TABLE I
The Evolution of 28 S Ribosomal Genes in Primates[a]

Species compared	T_m (°C)	T_m difference (°C)	% Diverged[b]	Years × 10^{-6} Diverged[c]	% Divergence per 10^6 years
Human-Human	85.5	0	0	0	0
Human-Chimpanzee	83.5	2.0	1.33	30	0.044
Human-Baboon	82.0	3.5	2.33	60	0.039
Human-Marmoset	80.0	5.5	3.66	90	0.040

[a]Human 28 S ribosomal RNA was hybridized under optimum temperature conditions with genomal DNA from each of the species shown, and the T_m's of the heteroduplexes compared with that of the homoduplex. There is a progressive and approximately linear increase in ΔT_m with increasing evolutionary distance. (From Jones and Purdom, 1975.)

[b]1.5°C; T_m = 1% altered base pairs.

[c]Simons (1964).

(Jones and Purdom, 1975) suggest that the 28 S cistrons in primates are diverging at about the same rate as that calculated (Kohne, 1970) for the unique genome of primates. This steady rate of evolution may make measurements of the divergence of ribosomal cistrons a useful approach to determining tentative divergence times among groups where the fossil data are fragmentary. At the present time, however, evidence concerning the evolution of these classes of repetitive DNA in primates is sparse, although certain of them have been mapped on primate chromosomes (e.g., Henderson *et al.*, 1972, 1974; Evans *et al.*, 1974; Warburton *et al.*, 1975).

To understand the complexity of the processes underlying the general picture as shown from comparative Cot analysis, the evolution of the repeated genome must be considered in the light of the finding that repeated and nonrepeated nucleotide sequences are intimately interspersed.

V. LINEAR ORGANIZATION OF THE GENOME

The patterns of interspersion of nonrepeated and repeated sequences along the DNA molecule is best known for the genomes of *Xenopus* (e.g., Davidson *et al.*, 1973) and sea urchin (Graham *et al.*, 1974) but recent work (Schmid and Deininger, 1975) shows that the human genome is substantially similar to these better known examples.

At least 25% of the human genome consists of an interspersion of short single copy sequences with an average length of 2 kb (kb = kilobase = 1000 bases) with short repetitive sequences which have an average length of 0.4 kb. A second portion of the genome is also occupied by an interspersed sequence arrangement

characterized by longer single copy sequences of unknown length. These two patterns, together with any regions of tandem repetition, occupy a minimum of nearly 80% of the genome. There is a 5% class of inverted repeated sequences, called foldback or zero order DNA, the structure of which is arranged such that a short base sequence is covalently linked to its complement, permitting intra-strand base pairing within single strand fragments. Its function, like that of most repeated DNA, is unknown but it is found throughout the genome (Wilson and Thomas, 1974). It is present in heterogeneous nuclear RNA (Jelinek *et al.*, 1974; Molloy *et al.*, 1974; Ryskov *et al.*, 1972), but absent from mature messenger RNA (Ryskov *et al.*, 1972).

Corneo and his colleagues have isolated two repeated DNA fractions from human total DNA renatured at intermediate values of Cot (Ginelli and Corneo, 1976). These components have been called fast intermediate DNA (buoyant density 1.703 gm/cm^3) and slow intermediate DNA (1.707 gm/cm^3) on the basis of their kinetic properties. Fast intermediate DNA has a complex organization; it is in part clustered in fragments larger than 24,000 nucleotide pairs, as a satellite-like fraction, and in part in fragments ranging from 1800 to 600 nucleotide pairs spaced with more complex sequences. Slow intermediate DNA is finely dispersed along the genome and can be isolated only by using DNA with a low fragment size (600 nucleotide pairs) and annealed to high Cot values. The fast intermediate DNA is probably not, or only poorly, transcribed *in vivo*, while the slow intermediate fraction is possibly transcribed to a great extent. The latter sequences are organized more like the *Xenopus* pattern (Davidson *et al.*, 1973) whereas the fast intermediate DNA are somewhat akin to satellite DNA in their tendency to clustering. Recent studies show that they are concentrated in C bands in human chromosomes (K. W. Jones, unpublished data).

The arrangement of most repeated DNA in chromosomes is unknown, but indications are emerging which suggest that the cytologically visible features of chromosomes may be based upon some property of repeated nucleotide sequences.

VI. REPEATED DNA AND CHROMOSOME STRUCTURE

Direct evidence suggesting that cytologically visible chromosome structure may be based upon particular classes of repeated DNA has been derived from hybridization *in situ* of radioactive probes complementary to pure fractions of DNA, satellite DNA's in particular. Satellite DNA appears to be concentrated essentially in constitutive heterochromatin, most of which may be arranged in bands (C-bands) close to the centromeric regions (e.g., Jones, 1970; Pardue and Gall, 1970) or in blocks occupying distal chromosomal regions. Alterations in highly repeated DNA therefore essentially concern changes in the heterochro-

matin structures in genomes, which are, judging from the scale of repeated DNA change, altering rapidly in primate evolution. As will be discussed, certain structural differences between the chromosomes of man and the chimpanzee may be correlated with the evolution of particular satellite DNA's.

Loss of ribosomal cistrons by mutation (Wallace and Birnstiel, 1966) is accompanied by the disappearance of a secondary constriction (Kahn, 1962) in *Xenopus* chromosomes. *In situ* hybridization of radioactive ribosomal sequence probes in primates (e.g., Henderson *et al.*, 1972; Evans *et al.*, 1974) shows these also to be associated with such chromosomal structural features. Loss and gain of repeated DNA therefore correlates with cytological changes and encourages the view that perhaps all visible chromosomal features are similarly based.

It may be inferred, from the interspersed nature of repetitive and unique nucleotide sequences, that chromosomes will have repeated sequences throughout their structure. This has been directly demonstrated by hybridizing radioactive copies of complex repeated DNA fractions to chromosomes *in situ* which shows them to be ubiquitous (Jones and Corneo, 1971; Arrighi *et al.*, 1971; Saunders *et al.*, 1972). In certain cases the pattern of hybridization given by complex fractions has been shown to be discontinuous and related to the cytological banding structure of the chromosomes (Sanchez and Yunis, 1974). It seems clear that the banded structures seen in chromosomes are enhanced rather than produced by the various pretreatments applied (McKay, 1973; Yunis and Sanchez, 1973). Banding may therefore be based upon some underlying organization of repeated sequences.

If human chromosomes are treated with nonsequence-specific nucleases such as micrococcal nuclease and DNase I, the banded appearance is abolished and they become difficult to stain. Rather surprisingly, however, restriction enzyme treatment "produces" the banded appearance (Fig. 3A) (Jones and Singh, 1977). This enhancement of pattern varies with different restriction enzymes, but all the restriction enzymes which are active reveal patterns which are similar to those obtained by more conventional approaches. The DNA in restriction-banded chromosomes is cut so extensively that chromosomes not affixed to slides prior to treatment are destroyed. DNA extracted from such banded chromosomes is extensively degraded when analyzed on agarose gels. The chromosome banding patterns obtained are sometimes partial, compared with more conventional methods, in that certain small chromosomes are featureless.

The patterns of restriction cuts can be revealed by repairing the DNA *in situ* using DNA polymerase I and radioactive nucleoside triphosphates appropriate to the known restriction sequences. The chromosomes are then labeled (Fig. 3B) whereas nonrestricted control chromosomes are not labeled. This shows, as might be expected, that restriction sites are extensively distributed. However, certain regions, C-bands in particular, are not as intensely labeled as others and there is a discernible banding of the autoradiographic grain pattern (Fig. 3B), indicating a greater concentration of available restriction sites in some regions.

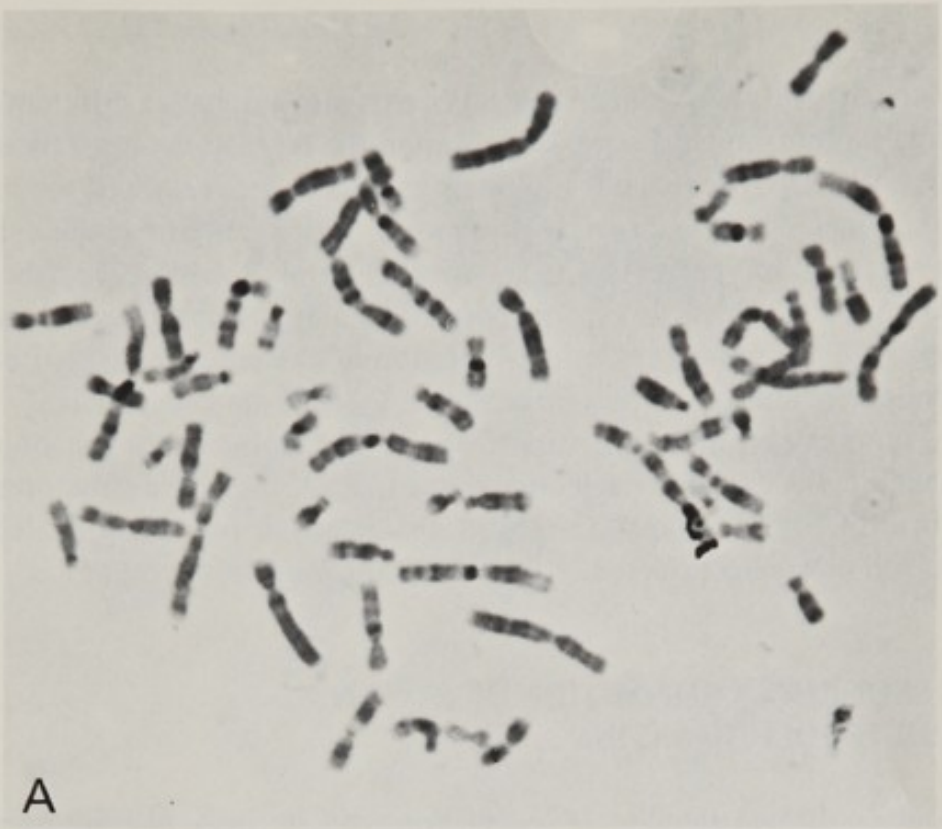


Fig. 3. (A) HeLa chromosomal banding following treatment with restriction enzyme (HAE III). The treatment emphasizes C-bands as well as bands along the chromosome arms. Homologues may be matched by their similar patterns. Other enzymes emphasize different aspects of chromosomal structure. (B) Human chromosomes used as substrates for DNA polymerase I after treatment with EcoRl restriction enzyme. DNA synthesis *in situ* is not random but appears distinctly localized in certain regions of the chromosome. Centromeric regions, for example, consistently lack label. Unrestricted chromosomes do not promote DNA synthesis (adapted from Jones and Singh, 1977).

The fact that different sequence-specific enzymes emphasize different aspects of banding patterns might be predicated upon the regional concentration of different repeated DNA sequence families as an underlying feature of the patterns. In chromosomes, such as those of the mouse which contain satellite DNA with known restriction properties (e.g., Southern and Roizes, 1973) the expected high frequency of cuts in C-bands, determined by repair replication, is not seen, however, ruling out a simple interpretation of the enhancement of banding by restriction enzymes at least as far as mouse C-bands are concerned.

In summary, certain structural features of chromosomes can be correlated with underlying repeated nucleotide sequences. Of these, the structures involving constitutive heterochromatin are best characterized in terms of satellite DNA. These will now be considered in the case of the human karyotype.

VII. SEQUENCE COMPOSITION OF HUMAN HETEROCHROMATIN

Four confirmed satellite DNA fractions can be isolated from human DNA. These have been called satellites I through IV in order of their discovery and isolation by Corneo and his colleagues (reviewed in Jones, 1973). Similar fractions have also been isolated by kinetic methods (Marx *et al.*, 1976) and other highly repeated components have been described by Saunders *et al.* (1972). Satellites I, II, III and IV have been mapped by *in situ* hybridization studies on human chromosomes (see Jones, 1973; Jones *et al.*, 1974; Gosden *et al.*, 1974; Moar *et al.*, 1975). The currently known distribution of these is summarized in Ruddle and Giblett (1974). All are situated either in C-band heterochromatin or in the heterochromatic distal portion of the Y chromosome which are thereby partially sequence defined. Those chromosomes possessing the largest C-bands, No. 1, 9, 16 together with the Y distal heterochromatin, also possess the highest concentration of satellite sequences. There are, apparently, no exclusive locations for any known satellite DNA, and all C-band and Y heterochromatin appears to be composed of contributions from more than one sequence rendering sequence-specific functions rather improbable. However, the concentration of a particular sequence may predominate in a given location. Satellite II appears to be predominantly in the C-band of chromosomes 1 and 16 (Jones and Corneo, 1971) whereas satellite III appears to be predominant in the C-band of chromosome 9 (Jones *et al.*, 1973) and satellite I in the distal heterochromatin of the Y chromosome (Fig. 4) (Jones *et al.*, 1974). The C-bands of chromosomes 1, 9 and 16 and the Y distal region are notably polymorphic however (Starkman and Shaw, 1967; Craig-Holmes and Shaw, 1971) in that variations in the size of the bands occur in individuals. These may be correlated with greater or lesser concentrations of satellite DNA (Jones and Corneo, 1971).

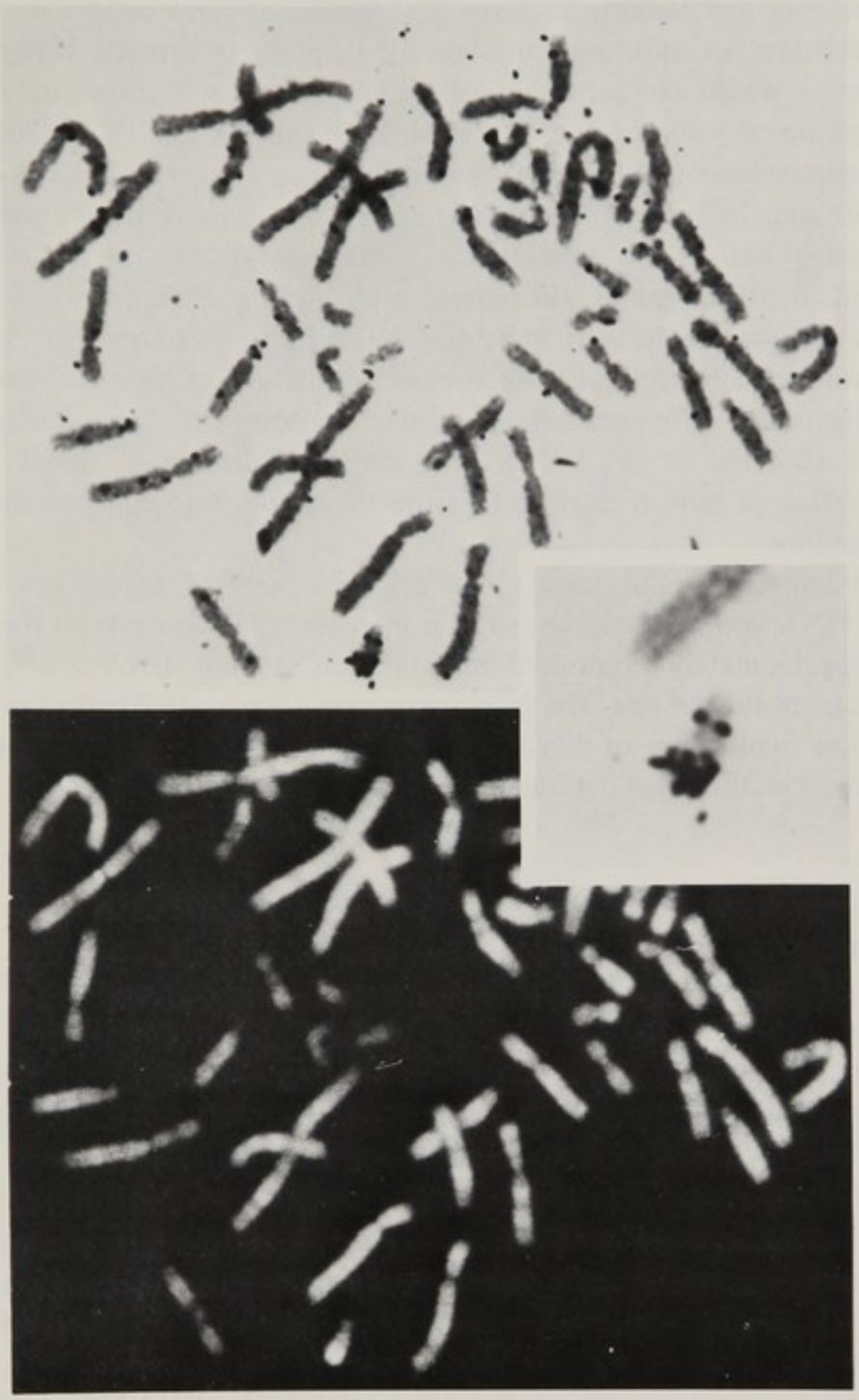


Fig. 4. The location of high concentrations of human satellite DNA I is evident close to the centromere (inset) as well as on the distal fluorescent portion of the Y chromosome, as can be seen from comparing the stained (top) and fluorescent (bottom) versions of the same human male chromosome spread. This feature is absent from the chimpanzee Y (see text), as is the satellite fraction concerned, and hybridization of satellite I cRNA *in situ* was negative. The appearance of new satellites or the translocation of existing ones in evolution thus correlates with the development of novel chromosomal structures. A similar situation pertains in respect of human satellite II (see text).

It is therefore not possible to make generalizations concerning the mapping of these fractions. An additional problem for mapping *in situ* arises from the cross reassociation which can occur between satellite DNA's. Human satellite DNA II will hybridize to some extent with satellite III (Melli *et al.*, 1975), although this can be controlled by conducting hybridization under very stringent conditions (cf. Jones and Corneo, 1971; Jones *et al.*, 1973). It seems likely, however, that estimation of the precise contribution of each sequence to the heterochromatin of individual chromosomes will require application of methods which will allow the isolation and analysis of individual human chromosomes, for example, by cell hybridization techniques. An interesting feature of human C-band heterochromatin is the differential staining which occurs with Giemsa when used at high pH (Bobrow *et al.*, 1972). This seems to distinguish bands with high concentration of human satellite III from those with high concentrations of the other satellites.

Some human chromosomes, 6, 8, and 11 for example, appear not to possess satellite DNA in *in situ* studies, so that man stands in contrast to the mouse in which approximately equivalent amounts of satellite DNA are seen on all chromosomes except one, the Y chromosome (Jones, 1970; Pardue and Gall, 1970). The significance of this is unknown, as is the significance of polymorphic variations in satellite-rich heterochromatin.

VIII. SATELLITE DNA HOMOLOGY WITHIN THE HIGHER PRIMATES

The presence of several satellite DNA's in the human genome poses the question of possible relatedness with satellite DNA's of the other higher primates. This question has been partially answered by cross hybridization using human satellite cRNA's. Human satellite III cRNA hybridizes *in situ* with C-band heterochromatin in the chimpanzee, gorilla and orangutan (Jones *et al.*, 1972). This finding correlates well with the Giemsa pH 11 staining reaction which also characterizes the C-bands of these species (Bobrow and Madan, 1973). The chromosomes of the gibbon, however, do not appear to show homology with the rest of the group in these respects.

There is a strong resemblance in human satellite III cRNA hybrid distribution between the karyotype of the gorilla and the orangutan after heterologous hybridization (compare Figs. 5 and 6). Both show strong cross reactions in the C-bands of the acrocentric chromosomes. The chimpanzee by comparison shows a distribution involving more of the metacentric chromosomes. This suggests a more conservative karyotypic evolution, in this respect, in the former species, despite the fact that the chimpanzee and gorilla are suggested to be more closely related on the basis of detailed karyology (e.g., Turleau *et al.*, 1972). Hetero-

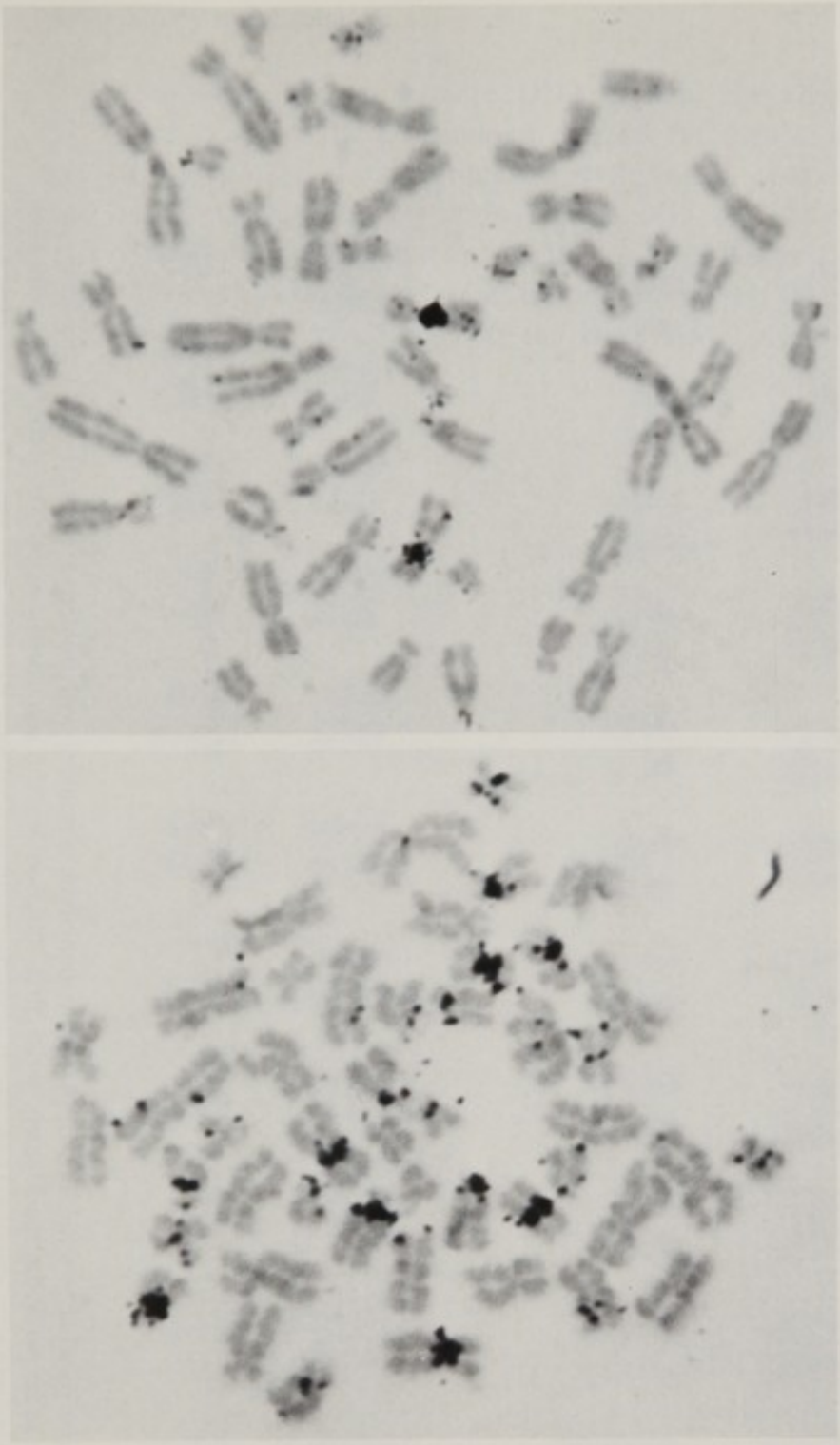


Fig. 5. The conservation of a satellite DNA in evolution is illustrated by these two chromosome spreads. The upper one shows the hybridization pattern of human satellite DNA III with human chromosomes where it localizes strongly on the No. 9 pair. The lower shows a chimpanzee chromosome spread hybridized under identical conditions. It is evident that there are related sequences present which are also in centromeric heterochromatin, but which are more prevalent and widespread. Human satellite III-related sequences are also found in the gorilla and the orangutan (see Fig. 6 and text). (Adapted from Jones *et al.*, 1972.)

Fig. 6. The upper panel shows the karyotype of the orangutan hybridized with human satellite III cRNA and arranged in descending size order with the metacentric series first, followed by the acrocentric series. The latter series exhibit a strikingly uniform pattern of a satellite DNA which is related to human satellite III. The lower panel shows the karyotype of the gorilla hybridized and arranged in a roughly similar fashion. It is obvious that in this case the acrocentrics also have a related satellite DNA which is also present on three or four metacentric pairs. When compared with the lower panel in Fig. 5, it is obvious that, in this respect, the gorilla and orangutan are quite different from the chimpanzee.

duplexes formed between human satellite DNA III and the DNA of man, chimpanzee, and orangutan show a decreased thermal stability in that order, consistent with the general belief concerning the evolutionary distance between these species (e.g., Goodman, 1962) (Fig. 7). Gorilla DNA has not been examined in this respect. Heteroduplexes between satellite III cRNA and the DNA's of lower primates, for example, the baboon, do not distinguish them from the DNA of mouse or bacteria, indicating that the sequences concerned are not

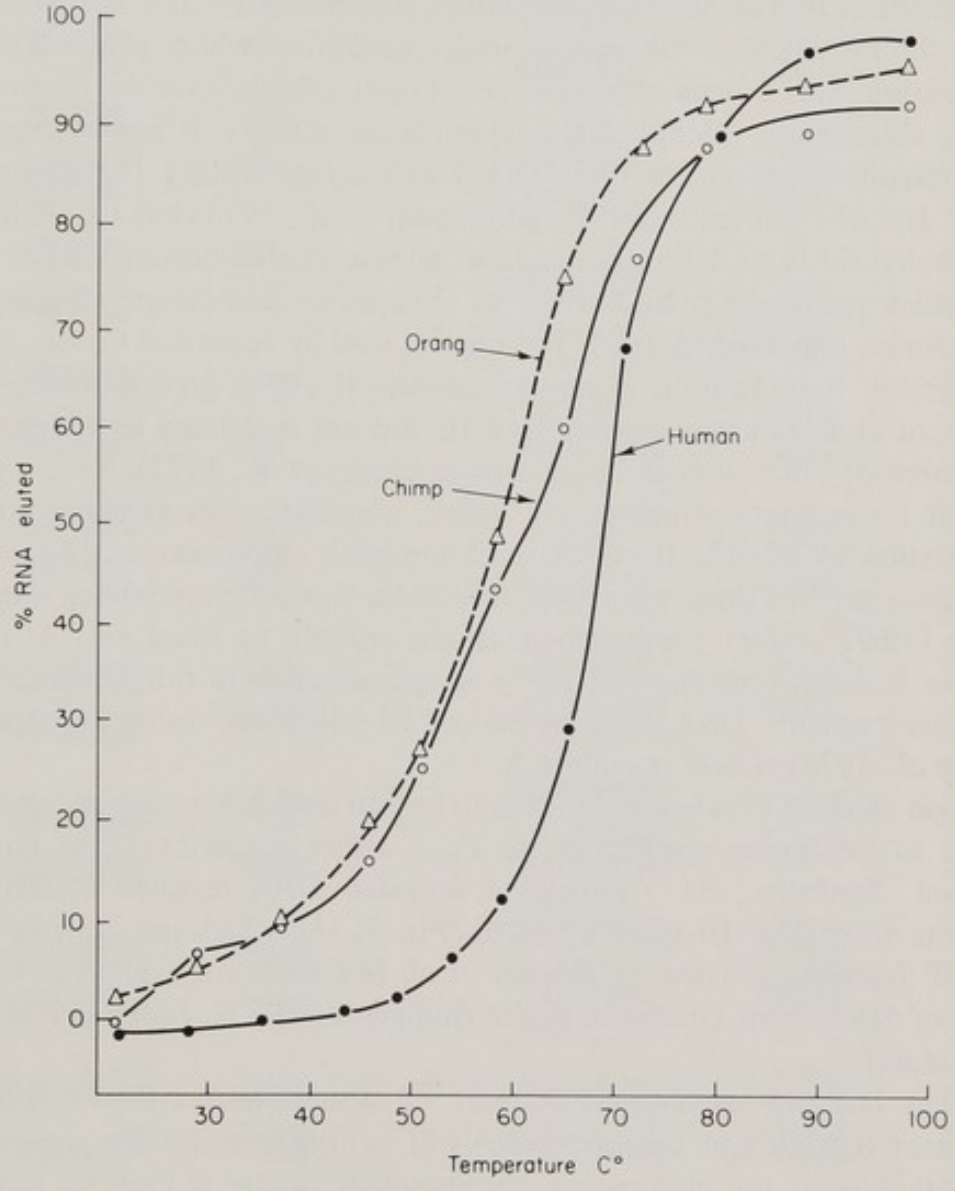


Fig. 7. Melting curves of RNA/DNA hybrids formed between human satellite III cRNA and the DNA of man, chimpanzee and orangutan. The hybridizations were taken to 80% saturation at the T_{opt} in 3X SSC (58°C). The T_m's were found to be 70°C for human (homologous hybrid); 63°C for chimpanzee; and 59°C for orangutan. The decreasing T_m suggests increasing sequence divergence with increasing evolutionary distance from man. (From Jones and Purdom, 1975).

present in detectable amounts in species further away from man than the orangutan (Jones and Purdom, 1975).

The higher primate DNA which cross-hybridizes with satellite III has been isolated as a DNA satellite (satellite A) from chimpanzee DNA. This satellite is very similar in all respects to human satellite III (Prosser, 1974) and its cRNA will cross-hybridize *in situ* with the same C-bands in man (Fig. 8) (Jones *et al.*, 1972).

Human satellite DNA I cross-hybridizes very slightly with interphase nuclei of the chimpanzee *in situ*, but definitive hybrid concentrations have not been noted on the chromosomes of this species under conditions which permit extensive hybridization with human chromosomes. Lower criteria have not been examined. A chimpanzee satellite DNA, chimpanzee satellite B has been isolated which resembles human satellite DNA I in buoyant density (1.686 gm/cm^3, satellite I; 1.683 gm/cm^3, satellite B) (Prosser *et al.*, 1973) but its cRNA does not cross-hybridize to definitive locations on human chromosomes under conditions which permit its hybridization to chimpanzee acrocentric chromosomes (K. W. Jones, unpublished data). The criteria used by Jones and Corneo (1971), which permit hybridization of human satellite II cRNA predominantly to the C-bands of human chromosomes 1 and 16, did not reveal any hybridization to chimpanzee or other primate chromosomes (Jones *et al.*, 1972). Recent experiments at lower temperatures (K. W. Jones, unpublished data) reveal extensive hybridization of satellite II cRNA to chimpanzee chromosomes. However the chimpanzee genome does not appear to possess a satellite resembling satellite II of man (1.693 gm/cm^3) although it cannot entirely be ruled out. At present therefore it seems probable that the cross-hybridization in this instance is with chimpanzee satellite DNA A, since human III which also cross reacts with II (Melli *et al.*, 1975) closely resembles A.

Human satellite IV when hybridized to human and chimpanzee chromosomes appears to be human-specific at its T_{opt}. Other conditions have not been examined. Similarly, the Y-associated repeated DNA revealed in restriction fragments with HAE III (Cooke, 1976) (Fig. 9) and which may correspond to the HAP fractions isolated by Kunkel *et al.* (1976) is not evident in HAE III digests of DNA from either sex in the chimpanzee (K. W. Jones and L. Singh, unpublished).

Human fast intermediate DNA, part of which isolates as a satellite-like component (Ginelli and Corneo, 1976) will hybridize under the same criteria with both human and chimpanzee chromosomes, where it localizes in C-band heterochromatin.

It seems probable that human satellite DNA II has derived from part of the diverged satellite III sequence by the sort of mechanism suggested by Southern (1970) (but, see also Smith, 1976; Salser *et al.*, 1976) in which satellite DNA's evolve by cycles of selection, amplification and divergence from pre-existing

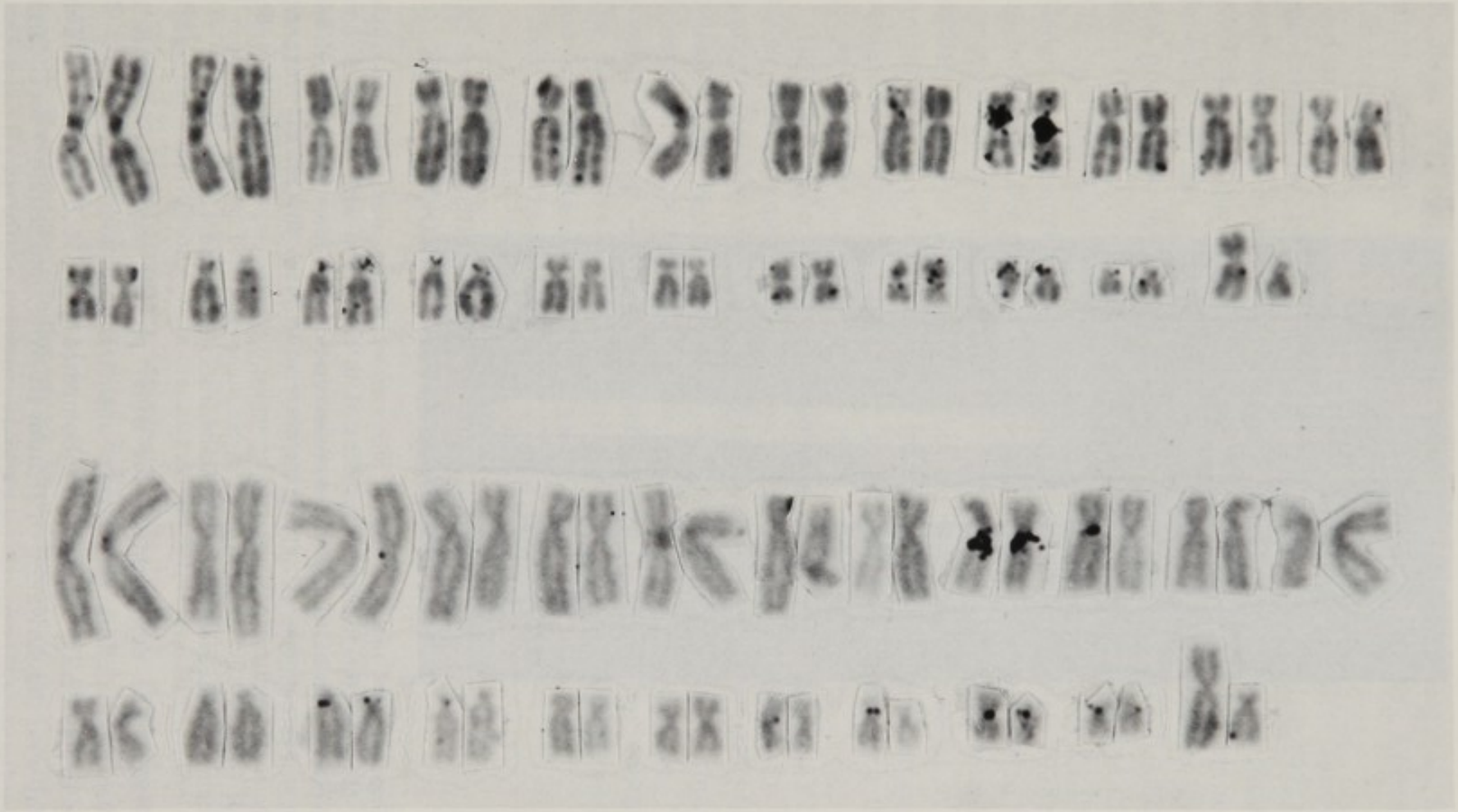


Fig. 8. Two human karyotypes. The upper one has been hybridized with human satellite III cRNA and the lower with chimpanzee satellite A cRNA. Both cRNA's hybridize preferentially with human chromosome pair 9, thus demonstrating their sequence homology. Likewise both satellite cRNA's hybridize indistinguishably with chromosomes of the orangutan. (Adapted from Jones *et al.*, 1972.)

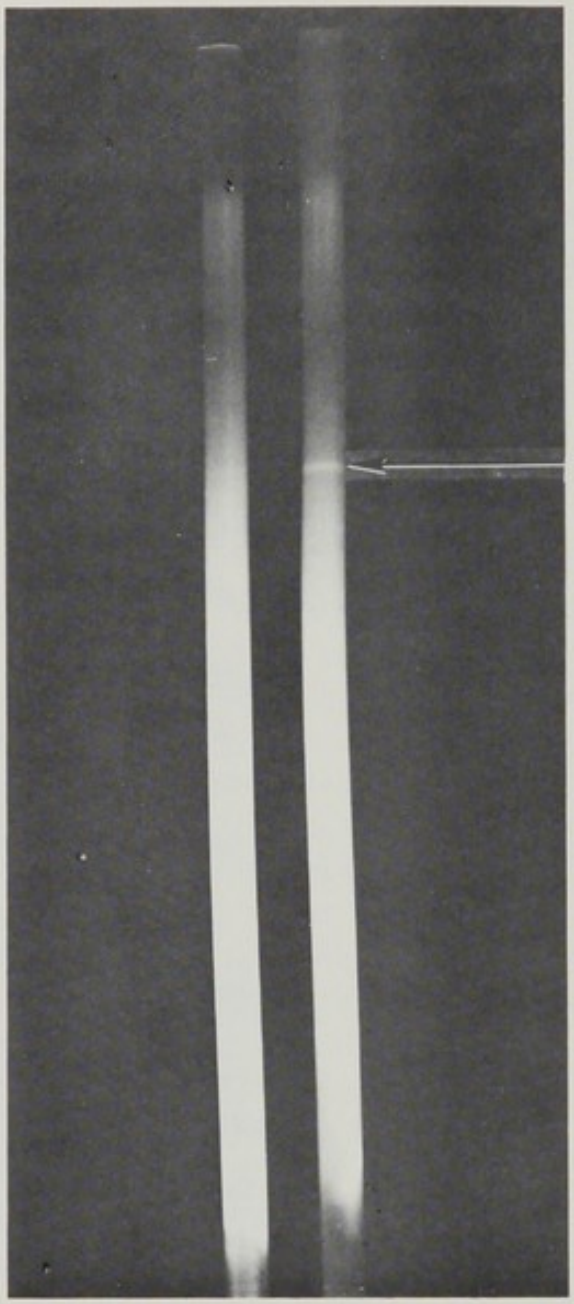


Fig. 9. Sex-associated repeated DNA in man (arrow). Human male (right) and female (left) DNA obtained from placentas was digested to completion with restriction endonuclease HAE III. Origin of gel on top. The products were separated on 1% agarose gels and stained with ethidium bromide. This phenomenon was first shown by Cooke (1976) and is confirmed here. Its significance is suggested by similar discoveries in snakes and birds (See text and Figs. 11, 12A,B) where there is sex chromosome-associated satellite DNA, which in snakes correlates with the evolution of chromosomal sex determination.

satellite DNA's. The derivation of the other primate satellite sequences is uncertain because there is no evidence yet of their interrelatedness.

Satellite III has a ΔT_m of about 10°C compared with 2°C for satellite II. This is consistent with the derivation of II from III by a rapid saltatory mechanism according to the model of Britten and Kohne (1968). Satellite DNA I has a ΔT_m

of about 6°C suggesting an intermediate extent of divergence. Human fast intermediate DNA has an estimated ΔT_m of 10–11°C (G. Corneo, personal communication).

Absence from chimpanzee chromosomes of the cytological features (Turleau and de Grouchy, 1972; Lin *et al.*, 1973; Bobrow and Madan, 1973) associated with satellite II in man, the absence of a similar satellite fraction from chimpanzee DNA, and the small ΔT_m of satellite II suggest that it arose substantially following the divergence of the ancestors of man and chimpanzee. Similarly, the lack of definitive hybridization between human satellite DNA I and chimpanzee DNA (Prosser, 1974) and chromosomes (Jones *et al.*, 1972), and the fact that the chimpanzee Y chromosome lacks the heterochromatin (e.g., Bobrow and Madan, 1973) associated with this satellite in man (Jones *et al.*, 1974) suggest its origin substantially subsequent to the same divergence, assuming no loss. In this respect however it is intriguing that the gorilla Y does show heterochromatin similar to that of the human Y, but nothing is known about its molecular structure. Lack of hybridization of human satellite IV with chimpanzee is also consistent with a later origin, but nothing is yet known about the melting properties of this satellite.

There are evidently strong homologies between the satellite DNA's of the higher primate group as shown by hybridization. This approach is however limited, since it is known that satellite DNA's may be as much as 71% related by sequence (i.e., two bases different in a repeating heptamer) without showing evidence of cross-hybridization (see Gall *et al.*, 1973; Blumenfeld, 1973). It is possible therefore that all of the higher primate satellites are sequence related. The fact that certain of them cross-hybridize slightly if at all however suggests that they have arisen from separate saltatory processes.

IX. RATE OF ADDITION OF SATELLITE DNA TO THE HUMAN GENOME

Based upon the observation that human satellite III-related DNA is found in the four higher primates, but is absent from the gibbon, it may be assumed to be $30–40 \times 10^6$ years old (Simons, 1964) (Fig. 10). Accepting that human satellite I, II, and IV have arisen substantially later, a rough idea may be formed of the average rate of addition of satellite DNA to the human genome. Estimations of the genomal contribution were made by satellites I, II, and III from their reassociation kinetics (Table II) total 1.54% (Jones, 1974). Satellite IV has been estimated to represent about 2% (Corneo *et al.*, 1972) making a total of 3.54% over 30–40 million years. This is likely to be a minimal value since it does not take into account other possible satellite DNA's. Measurements of the rate of addition of undiverged DNA to the genomes of primates by kinetic means however have

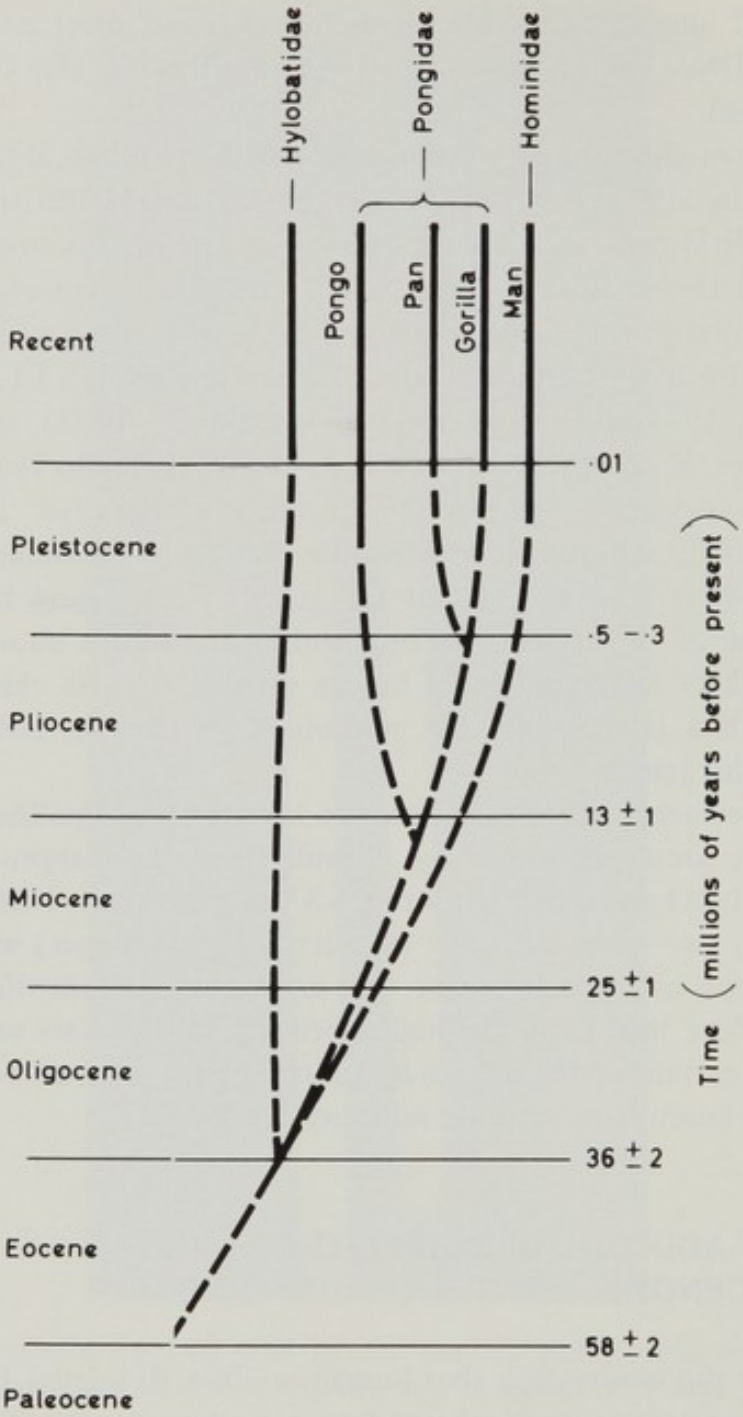


Fig. 10. A simplified diagrammatic representation of the phylogeny of the higher primates based on Simons (1964). The broken lines show hypothetical evolutionary relations. In the interval between the Eocene and Miocene times, these relations are particularly uncertain.

yielded similar values. Hoyer and Roberts (1967) measured the extent of homology between repetitive DNA's of human and primates at relatively low criteria in the DNA-agar technique. Kohne (1970) subsequently used these data to calculate the average rate of genome growth in primates as 0.07–0.09% of the human genome per million years since the appearance of the Anthropoidae.

TABLE II
Kinetics of Human Satellite DNA's[a]

Satellite	$T_{0.5}$ (3 μg/ml)	% of genome
I	48 seconds	0.14
II	24 seconds	0.6
III	16 seconds	0.8
IV	–	2.0[b]
		3.54

[a]Complexity and genomal percentage of human satellite DNA's. The values for satellites I, II and III were determined by rate hybridization at optimum temperature (T_{opt}) in 6× SSC-30% formamide (adapted from Jones, 1974).

[b]The value for satellite IV is taken from Corneo *et al.* (1972).

Similar measurements (Gummerson, 1972) under stricter criteria using HAP thermal chromatography gave comparable results with rates of 0.07–0.15% per million years (Table III). This has been interpreted to mean that genome growth occurs essentially by the addition of undiverged sequences (Gummerson, 1972). The coincidence between the values calculated and those estimated in respect of satellite DNA addition over a similar period further suggests that it is this form of DNA which accounts for the bulk of sequences added, and that the mechanism for this resides in centromeric heterochromatin.

TABLE III
Comparison of the Data of Gummerson (1972) and of Kohne (1970) on the Rate of Addition of DNA to the Genomes of Primates[a]

Animals Compared	% Undiverged sequences not held in common (% of genome)	Years since Divergence ($\times 10^{-6}$)	Gummerson's % of present genome added as undiverged sequences per 10^6 years	Kohne's % of present genome added as repeated DNA per 10^6 years
Human-Chimpanzee	2.25	15–30	0.15–0.075	–
Human-Gibbon	2.35	30	0.078	0.07
Human-AGM[b]	4.6	34	0.102	0.09
Human-Baboon	3.75	45	0.084	0.09
Human-Rhesus	5.17	45	0.115	0.09
Human-Slow *Loris*	7.18	75	0.104	0.28

[a]There is close agreement on the value of 0.11–0.12%, on the average, of DNA being added to the human genome every million years. This value is similar to that calculated for the rate of addition of satellite DNA (see text). (From Gummerson, 1972.)

[b]AGM, African green monkey.

Translocation of satellite DNA to other heterochromatin on nonhomologous chromosomes does not appear to occur at random. The mouse Y chromosome, which alone possesses no detectable satellite DNA, suggests that the process of spread may be determined by functional parameters. This suggestion is supported by the reverse situation in which a particular satellite DNA is essentially concentrated on the sex chromosome alone. The best examples of this are not in primates but they are of importance for our understanding of the evolution of sex chromosome-associated repeated DNA which occurs in man (Cooke, 1976; Kunkel *et al.*, 1976).

X. SEX CHROMOSOME-ASSOCIATED REPEATED DNA

The evolution of sex chromosomes in mammals has been accompanied by conservation of the linkage groups concerned (Ohno, 1973) as well as by the conservation of the chromosome banding pattern (Pathak and Stock, 1974). Sex chromosomes provide a means to analyze the DNA of a single chromosome of known function, from comparison of the repetitious DNA's of the sexes and of sex chromosomes with autosomes.

Sex chromosome-associated repetitive DNA has recently been found in studies on snakes and birds (Singh *et al.*, 1976) (Fig. 11). These have female heterogamety based upon the so-called ZW/ZZ system of chromosomal sex determination. The primitive snakes, represented by the pythons, show no evidence of chromosomal heteromorphism in contrast to the highly evolved snakes, including the highly poisonous varieties, which show well marked, often dimorphic, sex chromosomes. Analysis of satellite DNA's in a series of snakes, representing increasing specialization in this regard, showed a pattern of increasing elaboration ranging from single inconspicuous satellite DNA'S to multiple, highly conspicuous, satellite DNA's. Forms with differentiated sex chromosomes show a difference in female DNA which possesses a satellite which is much reduced or absent from male DNA. By *in situ* hybridization, the sex-associated satellite maps to the W chromosome with no concentrations of the sequence evident elsewhere. The W-associated satellite DNA from one species will hybridize *in situ* with the W chromosome of another species (Fig. 12A) indicating conservation of the sequence. Moreover, hybridization of snake W-satellite cRNA to the DNA of birds shows twice the saturation value with female DNA compared with male DNA. Birds, similarly, possess W-associated satellite DNA (Fig. 12B) (Singh *et al.*, 1976). Conservation of the sex chromosomes therefore also involves the conservation of highly repeated DNA.

The role of this DNA in the evolution of sex dimorphism is suggested by experiments in which the cRNA of the W-associated satellite has been hybridized to chromosomes of a series of snakes beginning with species which show no

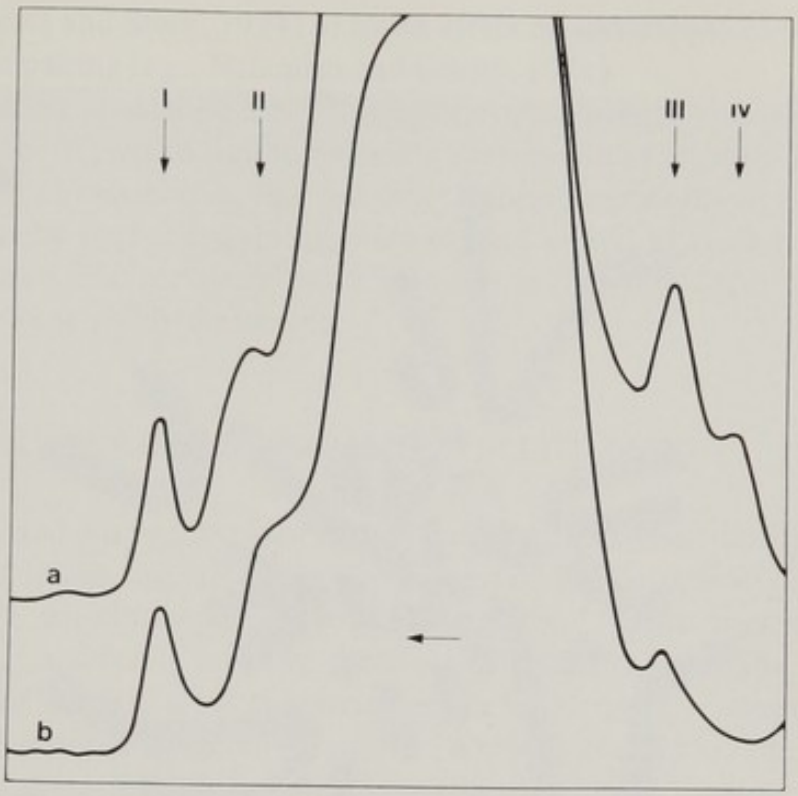
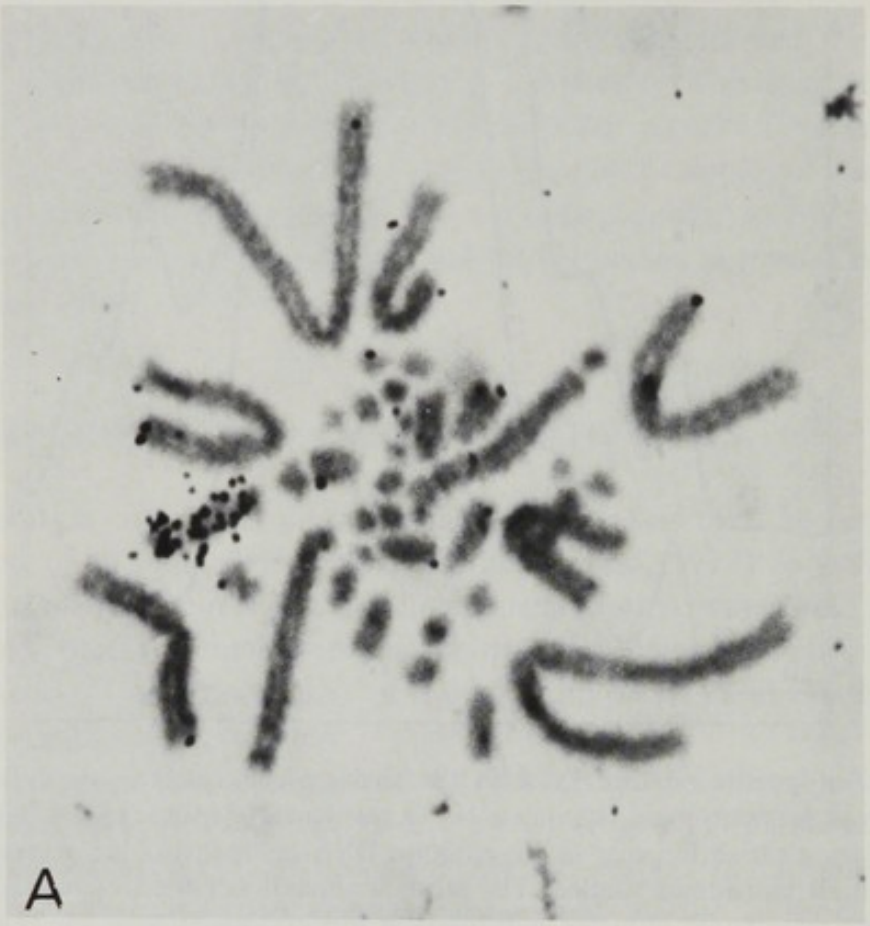


Fig. 11. Sex-specific satellite DNA in the snake *Elaphe radiata*. Analytical ultracentrifuge trace of (a) female and (b) male DNA in cesium sulfate silver ($Ag^+ = 0.20$) isopycnic gradients. Very clear differences in the satellite DNA components are evident, which are repeatable in all individuals studied. The most prominent satellite in females, satellite III, which is underrepresented in males, hybridizes *in situ* exclusively with the W chromosome of closely related species in which the sex chromosomes are clearly differentiated (see Fig. 12A). Sex-associated repetitive DNA has also been found in man (see Fig. 9) and may be a widespread phenomenon (see text). Arrow (←) indicates rotor center.

evidence of chromosomal sex determination. In these, there is no evidence of hybridization to any chromosome. Moreover, filter hybridization to the DNA of each sex does not distinguish them from controls of mammalian DNA. Intermediate forms, in which the sex bivalent is homomorphic, show hybridization only to a single chromosome, the W, and their DNA hybridizes to different saturation values according to sex. It seems clear from this that differences in the repeated DNA composition of the W chromosome precede the evolution of sex bivalent dimorphism. This suggests that these repeated DNA sequences are playing a role in the sex differentiation of snakes.

The probable mechanism of this is suggested by the fact that heterochromatin rich in satellite DNA frequently replicates with a different pattern from the rest of the genome (e.g., Ganner and Evans, 1971) and is associated with suppression and interference with crossing-over among homologues (e.g., Klasterska *et al.*, 1974; Thomas and Kaltsikes, 1974). Crossing-over might be influenced by the proximity of satellite DNA either through effects directly on the DNA synthesis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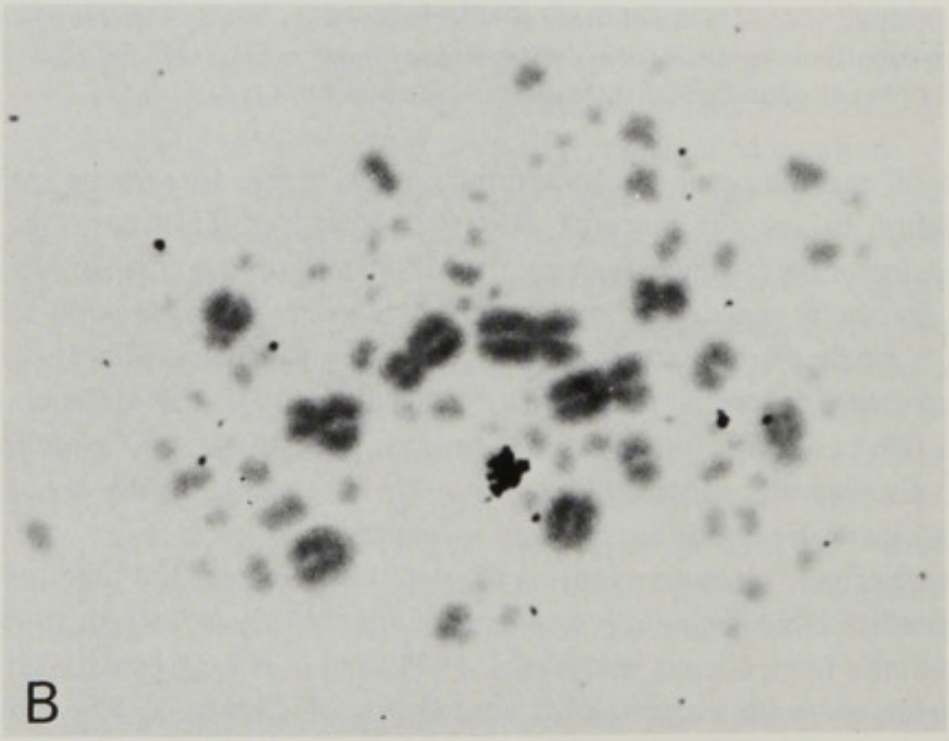
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involved (Hotta and Stern, 1974) or by an effect of asynchrony of replication on chromosome pairing (e.g., Natarajan and Gropp, 1971).

Translocation of satellite DNA throughout the chromosome, such as appears to occur in the W, would lead to complete suppression of crossing-over. Satellite DNA on such chromosomes, like the other sequences concerned, would then be less likely to be exchanged during meiosis, and would be conserved over long periods of time. The previously homologous bivalents would then evolve separately and become visibly dimorphic.

XI. SIGNIFICANCE OF AUTOSOMAL SATELLITE DNA

The localized arrangement of autosomal satellite DNA, usually in C band heterochromatin, is suggested, from consideration of the probable functions of such DNA in sex chromosomes, to reflect the fact that its presence will have some effect on crossing-over. In certain tissue culture lines, the centromeric localization of satellite DNA is relaxed, and intercalary heterochromatic blocks are often a feature of their chromosomes (White *et al.*, 1975). The presence of such blocks along the arms of autosomes is therefore not incompatible with cellular function, and it may simply reflect the absence of meiosis.

A deterrent effect of C-band heterochromatin on crossing-over would result in the relative stabilization of adjacent linkages compared to those on chromosome not possessing prominent C-bands. Reduction of crossing-over between such homologues would lead to a tendency for the combinations of genes immediately covalent to the C-bands to be transmitted more frequently as a unit. Accordingly the phenotypic features controlled by such linkages would show a relatively strong tendency to be inherited integrally and to behave as a unit with respect to natural selection. Such a mechanism would form the basis of intraspecies polymorphisms.

C-bands of different sizes (C-band polymorphisms) might be expected to exert more or less influence on the degree to which adjacent linkages were stabilized and therefore on the stability of the phenotype transmitted through

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Fig. 12. A. Sex chromosome-associated satellite DNA in snakes. *In situ* hybridization of sex-associated satellite DNA cRNA from *Elaphe radiata* (Fig. 11) with the chromosomes of *Natrix piscator* female. The W chromosome, which is morphologically distinct, is the only consistent site hybridized. It is notable that the satellite DNA sequences are spread along the entire length of the chromosome although the genomal DNA contribution of this chromosome exceeds that of the satellite DNA, suggesting interspersion with other DNA. Similar results have been obtained with another snake *Bungarus coeruleus*. (Adapted from Singh *et al.*, 1976). B. *In situ* hybridization of radioactive cRNA of a satellite DNA fraction from the domestic chicken to female chromosomes of that species. The W chromosome is heavily and exclusively labeled, indicating a sex-associated repeated DNA similar to that found in snakes (Singh *et al.*, 1976).

many generations. The adaptive desirability of the phenotypes concerned would decide the population frequency of a given chromosome bearing this type of structure as a function of evolutionary time. This would lead either to the conservation or to the elimination of the satellite DNA associated with that particular chromosome. The present day C-band polymorphisms in man may thus be interpreted in such an evolutionary context since they are known to be heritable (reviewed in Schwarzacher, 1976) and particular racial groups of man exhibit characteristic frequencies of particular chromosomal polymorphisms. The conservation of human satellite III-related sequences among the primates may reflect the stabilization of combinations of genes which have played an important role in the evolution of this group. Similarly the linkage groups on chromosomes 1, 9, and 16 of man may be those which have played a distinctive role in the evolution of man from his common ancestors with the primates.

The chromosomal locations of satellite DNA are also those which are involved in Robertsonian translocations. A very good example of this in evolution is seen in the case of the tobacco mouse (*Mus poschiavinus*) which has apparently evolved from *Mus musculus* by the occurrence of seven such fusions (Gropp *et al.*, 1970). Karyotypic evolution by chromosome fusion is frequent and it is possible to see that satellite DNA, because of its lack of transcriptional function, could potentiate it. There is an increased incidence of translocations involving prominent satellite DNA-rich heterochromatin in man (Hansmann, 1976). According to the present view of the role of satellite DNA, chromosomal fusion would be potentiated between those chromosomes which already had evolved to possess especially adaptive combinations of genes. The addition of highly repeated DNA to genomes can therefore be seen as a mechanism which stabilizes and integrates combinations of genes under the influence of the forces of natural selection. In this sense it is easy to appreciate why species are different in their satellite DNA content and why this type of DNA is evolving rapidly. According to the orthodox Darwinian view, species evolve from the polymorphisms present within a population. To the extent that it is involved in the processes discussed, therefore, satellite DNA plays a central role in speciation.

With these arguments in mind, it is interesting to speculate upon the possible role of particular satellite DNA's in the evolution of man.

XII. SATELLITE DNA AND HUMAN EVOLUTION

According to the evidence discussed earlier, there is reason to believe that the different human satellite DNA's may have arisen at different times in evolution. Satellite III appears to have accompanied the divergence of the higher primate ancestor from that of the modern gibbons at around 30–40 million years, whereas satellites I and II may have arisen subsequently. The average rate of

divergence of satellite III, from its 10° ΔT_m, is 0.33°–0.25° per million years. Assuming that the other satellites have diverged at approximately the same rate, satellite I (ΔT_m = 6°C) will be 24–18 million years old and satellite II (ΔT_m = 2°C) will be about 8–6 million years old. The palaeontological record (Simons, 1964; 1972) shows evidence of extinct forms of hominids which have been dated fairly precisely by potassium-argon methods. The two forms which are supposed to be more man-like and therefore to have arisen along his direct lineage are *Ramapithecus* and *Australopithecus* with dates of 14 million and 3–5 million years. These dates will very likely reflect the times at which these species achieved large enough populations to ensure much chance of discovery, rather than their origin. They therefore may correspond with the estimated ages of human satellite DNA's I and II well enough to permit the suggestion that these satellite DNA's may have played some part in the evolution of these ancestral species, perhaps along the lines discussed here.

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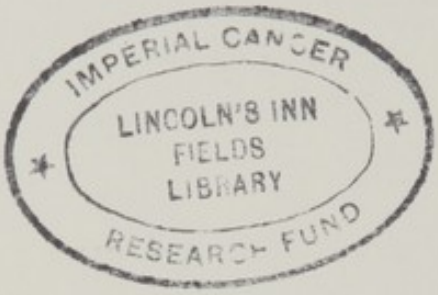

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