Genetic structure and function / P.F. Smith Keary.

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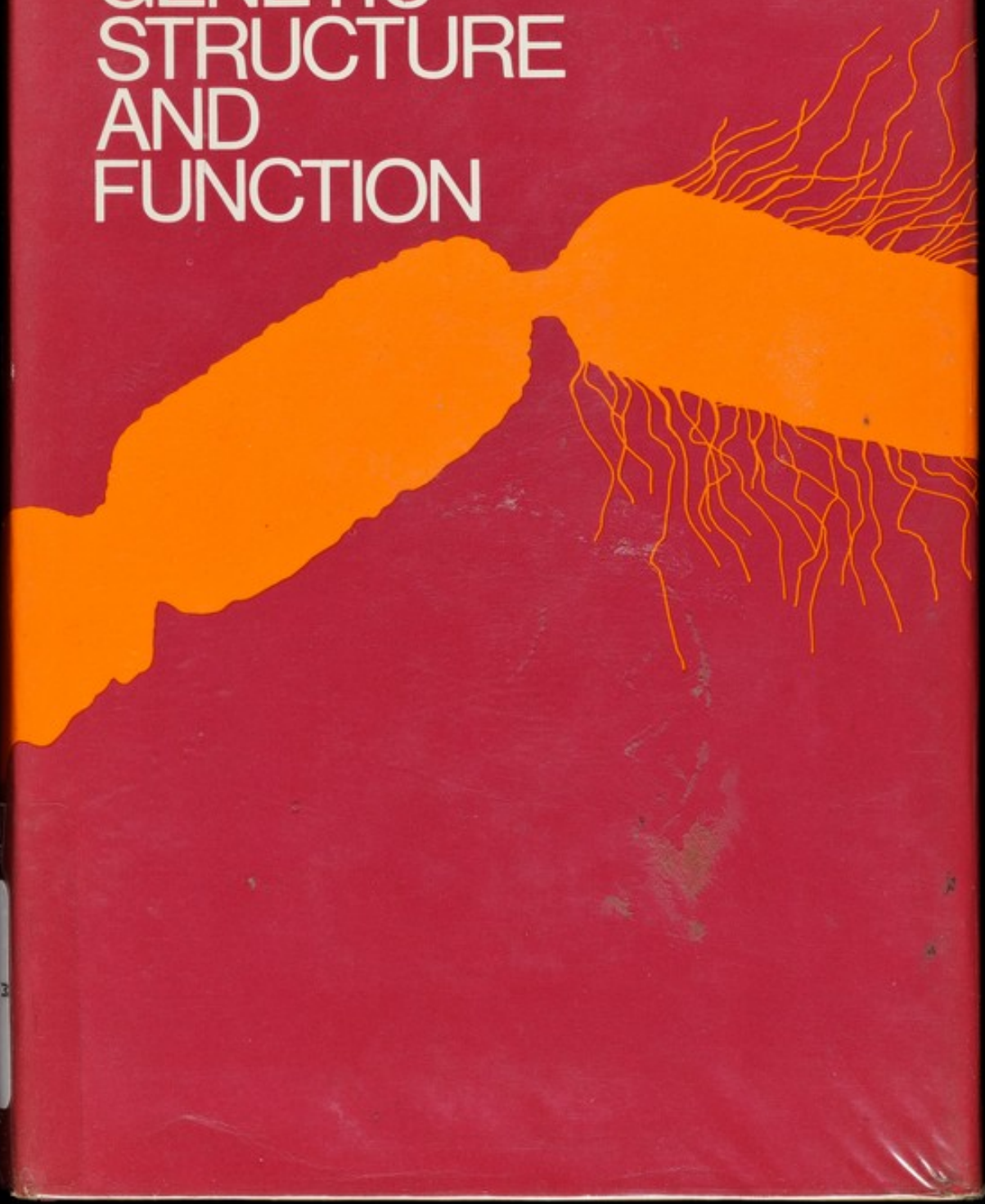
P F SMITH-KEARY

GENETIC STRUCTURE AND FUNCTION



JOHN McLEISH and BRIAN SNOAD
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Demonstrates, by means of a continuous series of photographs and illustrations, how the carriers of genetic information, the chromosomes, behave during the growth and reproduction of plants. The text has been thoroughly revised and incorporates much new information on the chemical components of cells, particularly of nucleic acids and proteins.

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General Microbiology, 3rd Edition

The third edition of this popular textbook has been rewritten to present a comprehensive treatment of introductory microbiology. Five new chapters are included on groups of bacteria and their properties. Microbial diseases are discussed in the context of symbiotic relationships, and in the case of host parasites in vertebrates there are sections on resistance, disease production and antibodies.

This book is designed to provide a full and up-to-date introduction to molecular genetics for university and polytechnic students taking a first course on the subject.

The subject matter of the book is the genetic material – its structure, function, organisation, replication, transmission, recombination and mutation. In addition, there are other topics, such as the relationships between genes and proteins, the genetic code, transcription and translation, and also the more formal aspects of genetics, all of which have a natural place in this book, and they have accordingly been treated in some detail. Genetic analysis is described in phage, bacteria and fungi, and there is a chapter on mutation which includes sections on how mutants are isolated, how they are induced and on the molecular basis of mutation. Among the many other topics described are the molecular basis of recombination, the repair of DNA, the *lac* operon of *E. coli*, gene suppression, mutation and cancer, position effects in *Drosophila*, controlling elements in maize and differential gene activity in eucaryotes.

In keeping with the great advances made by molecular biology in recent years, the book as far as possible discusses the genetic material from the molecular viewpoint. Another feature is the attention given to describing actual experimental techniques and results, so that the reader may be able, not only to evaluate the results for himself, but also to gain a deeper understanding of the logical foundations on which the subject rests.

For a biographical note on the author, please see the back flap

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Books cannot always please, however good;
Minds are not ever craving for their food.

George Crabbe (1754–1832)

Contents

Preface

This book was written partly because of an inner motivation and partly, and more important, because of an awareness of the lack of a suitable text for second and third-year courses in molecular genetics at Trinity College, Dublin. There are many good textbooks on general genetics but these tend to concentrate on Mendelian and neo-Mendelian genetics and lead on to a study of natural selection and evolution. On the other hand the text books on molecular genetics take a more biochemical approach and, in general, ignore many important aspects of basic genetics and genetic mapping, even though it is not possible, for example, to understand the rationale behind hybrid DNA models for genetic recombination unless one is fully conversant with tetrad analysis, or the relationships between the different control genes in bacteria without a knowledge of their genetic systems. This introduction to molecular genetics, based on lectures given to our second and third year students, is written from the point of view of a geneticist and attempts to achieve a better balance between the genetic and the biochemical evidence.

The size of any book limits the range of material that can be adequately described and so the choice of examples becomes largely a matter of personal preference. Several important aspects of basic genetics (for example inheritance in man, continuous variation and cytogenetics) receive only a brief mention since they are not necessary for a proper understanding of the aspects of molecular genetics described in this book. Likewise, only a few selected examples of genetic control in higher organisms are highlighted and some of the more important problems indicated. This is not because these systems are any the less interesting or less important than the better known control systems in procaryotes, but because they are not only strikingly different but also relatively poorly understood; many of the conclusions in this wide and rapidly expanding field are still tentative and cannot be adequately discussed in a book of this size and at this level.

In general, I have tried to trace the development of each branch of molecular genetics, to discuss each experiment in its correct historical perspective, wherever possible describing experimental procedure and presenting actual data or results, and to present a picture of molecular genetics as it is today.

I have not attempted to cite original references to all the experiments described, but the bibliography, in addition to some suggestions for further general reading, lists a number of now classic papers, which the student should find intelligible, informative and intellectually satisfying.

I am much indebted to the members of the Department of Genetics in Trinity College, Dublin, who have read and commented on many parts of the manuscript, to Dr Keith Jones, who prepared the photographs reproduced in figure 4.4. especially for this book, and to Dr Oscar Miller (jr) for permission to reproduce the electron micrographs shown in figure 18.6.

P. F. SMITH-KEARY

Trinity College,
Dublin
April 1974

Abbreviations and Symbols

A	adenosine
Ala	alanine
Ala-tRNA^{Ala}	alanine transfer RNA charged with alanine
AMP	adenosine 5′-monophosphate
AP	2-aminopurine
Arg	arginine
Asp	aspartic acid
Asn	asparagine
ATP	adenosine 5′-triphosphate
B–B′	prophage recognition sites on the bacterial chromosome
BUdR	5-bromodeoxyuridine
BU	5-bromouracil
C	cytidine
cAMP	adenosine 3′:5′-cyclic monophosphate
Cys	cystine
Δ	chromosomal deletion
d	prefix 'deoxy'
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
F_1	first filial generation
F	the *Escherichia coli* sex factor
fMet	formyl-methionine
G	guanosine
GTP	guanosine 5′-triphosphate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HA	hydroxylamine
Hb	haemoglobin
Hfr	high-frequency recombination
HNI	high negative interference
His	histidine
i	initiator site for replication
I	inosine
Ile	isoleucine
J	joule; practical unit of electrical energy
Leu	leucine
Lys	lysine

μm	micrometre (10^{-6} metres)
Met	methionine
MR-DNA	middle repetitive DNA
mRNA	messenger RNA
NA	nitrous acid
NG	N-methyl-N′-nitro-N″nitrosoguanidine
nm	nanometre (10^{-9} metres)
O	operator region
ϕ	bacteriophage
p	phosphate group
P	promoter region
pC, Cp	nucleotides of cytidine ending with a 5′ or a 3′ phosphate respectively
P-*P*′	sites on a phage chromosome that recognise *B*-*B*′
Phe	phenylalanine
Pro	proline
RG	regulator gene
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
S	Svedberg unit, the sedimentation coefficient
Ser	serine
SG	structural gene
T	thymidine
t	terminator
Thr	threonine
TMV	tobacco mosaic virus
tRNA	transfer RNA
$tRNA^{Ala}$	the transfer RNA for alanine (uncharged)
Trp	tryptophan
Tyr	tyrosine
U	uridine
uv	ultraviolet radiation
Val	valine

1 This is Genetics

Every schoolboy knows it.
Bishop Jeremy Taylor (1613–1667)

To most students of a few years ago, genetics would have conjured up the idea of ratios in peas or in the fruit fly, and even today a dictionary may define genetics as simply 'the science of the study of heredity'. Although we realise the importance of understanding how characters are inherited, the ramifications of modern genetics are very much greater and spread into all fields of conventional biology. Today, geneticists already know the detailed structure of at least one gene and how it can code for the production of a specific polypeptide molecule, and they are now seeking a finer knowledge of how the action of the genes themselves is controlled; with this knowledge we will be nearer to understanding how the multitude of genes in a higher organism coordinate their actions so as to be able to control the development of a fertilised egg into a complex multicellular adult organism. Genetics is probing at the very nature of life itself and, indeed, 'life' has already been synthesised in the test tube, albeit it by copying the comparatively simple form of an infective viral chromosome. Even more important socially is the possibility that in the not too distant future we may be able to replace defective genes and so alleviate the miseries caused by the many inherited and incurable diseases of man.

Although many who read this book will already have studied some genetics, others will not have done so, and it is to them that the next two sections are addressed; they introduce some of the many technical terms used in the text and outline some of the basic concepts and foundations of genetics. The remaining chapters assume that the reader has understood these terms and concepts.

A Mendelian View of Genetics

The fundamental unit of any higher organism is the cell and it is convenient first to examine the basic structure of a generalised animal cell (figure 1.1). Each cell is surrounded by a *cell membrane* about 7.5 nm thick, and like all the cellular membranes it is made up of a layer of phospholipid molecules sandwiched between two layers of protein molecules. This membrane is semi-permeable and so allows the passage of some macromolecules, but not others, and it is a barrier between the exterior and the interior of the cell. The cell membrane encloses the *cytoplasm* and within this lie a number of cell organelles, such as the mitochondria, lysosomes, centrioles, endoplasmic reticulum and the Golgi apparatus. The *mitochondria* consist of two layers of membrane with extensive internal invaginations; they are rich in enzymes and their function is to provide energy by the oxidation of food substances. *Lysosomes* are also membrane bound and they contain the enzymes concerned with the breakdown of

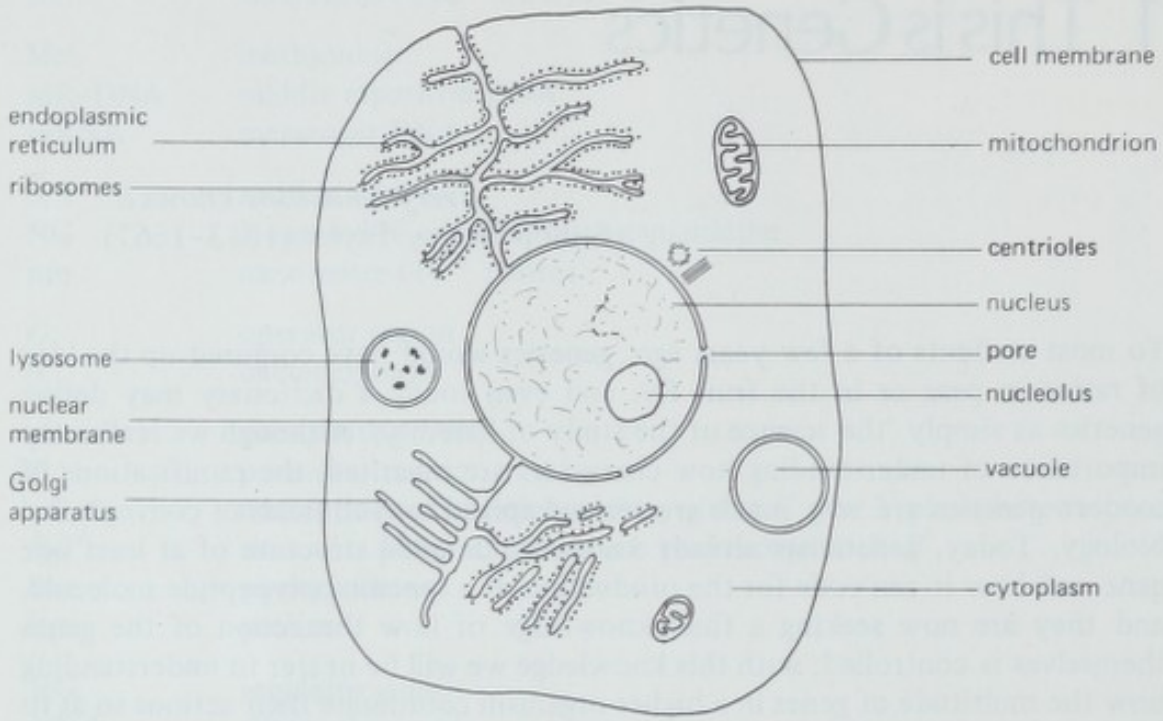


Figure 1.1 The generalised animal cell.

macromolecules. Every animal cell (but not plant cells) contains a pair of *centrioles* and they are responsible for organising the spindle at cell division (chapter 4). The *endoplasmic reticulum* is a system of membranes organised in pairs and it forms an intricate network within the cell. Some of the endoplasmic reticulum, the *rough* endoplasmic reticulum, has one surface lined with *ribosomes.* The ribosomes, made up of RNA and protein, are found either lining the endoplasmic reticulum or free within the cell and they are the factories where the proteins are synthesised; some of these proteins are secreted into the endoplasmic reticulum. Another membrane system, probably continuous with the endoplasmic reticulum, is the *Golgi apparatus*; this has a layered structure and there are no attached ribosomes.

The most important part of the cell is the *nucleus,* the control centre of the cell. The nucleus is also bounded by a double membrane, continuous with the endoplasmic reticulum, and it contains the genetic material (collectively referred to as *chromatin*) and one or more *nucleoli* where the ribosomal RNA is synthesised. The genetic information is stored in discrete bodies found in the cell nucleus, the *chromosomes.* Each chromosome is differentiated along its length into a very large number, perhaps 500 to 2000, of basic genetic units of *genes.* With certain exceptions each gene functions by specifying the biosynthesis of a particular polypeptide, often in the form of an enzyme, or by carrying out a control role in biosynthesis.

In higher organisms each cell usually contains *two* complete *sets* of chromosomes—in other words the chromosomes occur in pairs of two similar, but not necessarily identical, homologues, one derived from each parent. Each is

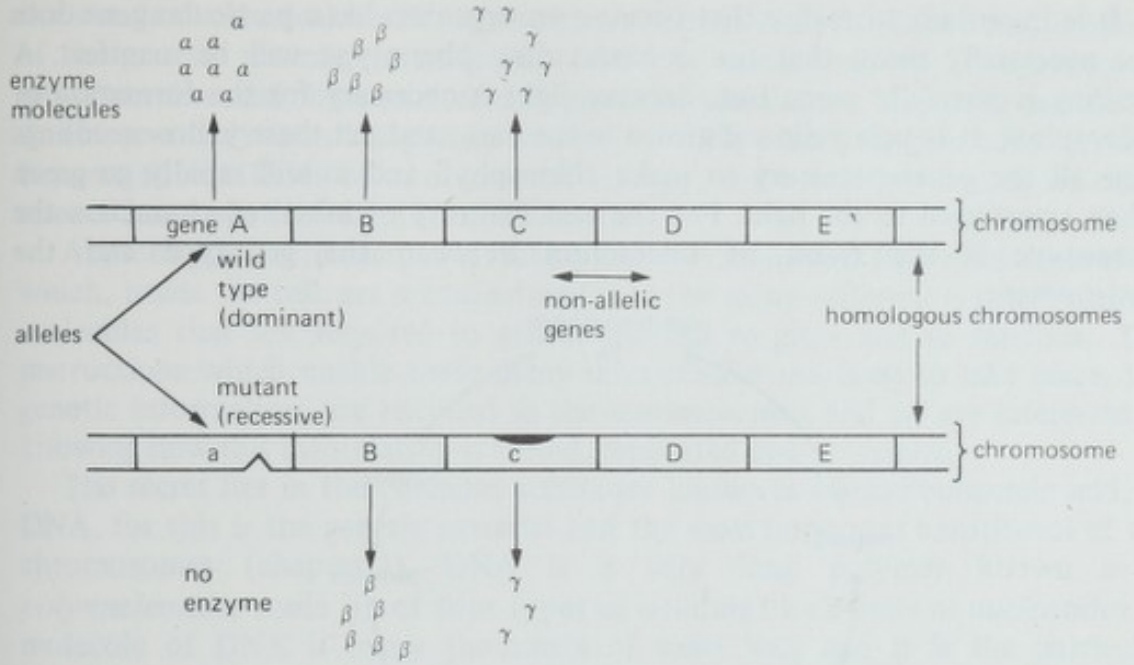


Figure 1.2 The organisation of the genetic material in a diploid eucaryotic cell.

a pair of *homologous* chromosomes. Along each chromosome the sequence of genes is identical although the genes themselves may be slightly different so that they produce qualitatively or quantitatively different products. In figure 1.2 one homologue has a defective gene *a* which has been structurally altered so that it can no longer specify the production of the α protein molecules, while gene *c* has been modified so that it produces fewer molecules of protein γ; both of the corresponding wild type genes, *A* and *C*, produce normal gene products. Thus *A* and *a* (or *C* and *c*) are different forms of the same gene, or *alleles* of each other, and in this example *A* is the wild type allele and *a* the mutant allele. *Mutation* is the general name given to the processes which can change one allele into another allele (chapter 10).

Since the chromosomes occur in pairs the genes must also occur in pairs, so that for the pair of alleles *A* and *a* there are three possible genic combinations or *genotypes*, *AA*, *Aa* and *aa*. When both homologues carry the same allele (*AA* or *aa*) the cell is said to be *homozygous* (and the organism is a *homozygote*), while if the alleles are different (*Aa*) it is *heterozygous*. In a heterozygous *Aa* organism the *A* allele will enable the production of the wild type protein and so the individuals will have a normal appearance or *phenotype* and be indistinguishable from an *AA* homozygote; the only individuals who will have the abnormal or mutant phenotype will be the *aa* homozygotes. We say that the *A* allele is *dominant* over *a*, or that *a* is *recessive* to *A*. Usually, but not always, the wild type allele is dominant and the mutual allele is recessive; dominant and recessive alleles are frequently denoted by capital and lower case letters respectively. Not all pairs of alleles show dominance and recessiveness and the heterozygotes may have a phenotype intermediate between the phenotypes of the two homozygotes.

It is important to realise that because an organism has a particular gene does not necessarily mean that the corresponding phenotype will be manifest. A seedling is normally green but, because light is necessary for the formation of chlorophyll, it is pale yellow if grown in the dark; and yet these yellow seedlings have all the genes necessary to make chlorophyll and so will rapidly go green when transferred to the light. For the vast majority of inherited characters the phenotype is the result of interaction between the genotype *and* the environment.

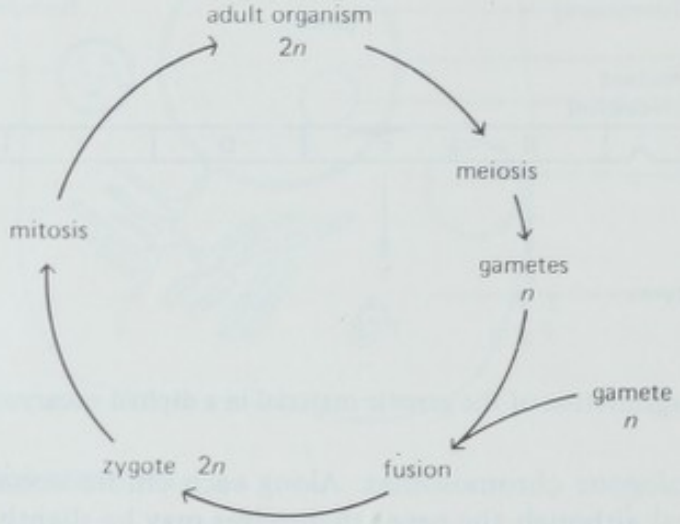


Figure 1.3 A generalised life cycle of a higher animal: n is the number of chromosomes in a haploid set; $2n$ represents a diploid cell or organism.

A cell containing two complete sets of chromosomes is called a *diploid* and a cell with only one set is *haploid*. The terms diploid and haploid are also used to refer to organisms containing predominantly diploid or haploid cells. The term *genome* is used to refer to the total gene content of a cell or organism.

A cell divides by the process of *mitosis* (chapter 4), in such a way that each daughter cell receives a complete and exact copy of all the chromosomes in the mother cell; it is by mitosis that a zygote divides so as to produce, eventually, a multi-cellular adult organism. However, in the life cycle of a sexually reproducing organism (figure 1.3) the diploid zygote is the product of the fusion of a male and a female gamete, and if the number of chromosomes is to remain constant and not to double at each successive generation there must, somewhere in the life cycle, be a special type of cell division which halves the number of chromosomes. This is *meiosis* (chapter 4) and it occurs during gamete formation so that each gamete is hapoid and contains only one set of chromosomes; when two gametes fuse to form a fertilised egg cell or *zygote* the diploid number is restored.

A Molecular View of Genetics

The ultimate aim of molecular genetics is to explain all of genetics, and indeed all of biology, in physical and chemical terms, the basis of the explanations being

the standard chemical bonds; it attempts to elucidate the structure and function of the genetic material in molecular terms by using a variety of sophisticated physical, chemical, biochemical and genetical techniques. Molecular genetics is therefore a truly interdisciplinary science, not only ramifying into but extending beyond all the fields of conventional biology, and in the last decade no other science has made such significant advances.

Any cell can be likened to a factory; there is an inward flow of raw materials which, inside the cell, are manufactured into the many different types of organic molecules that are required to enable the cell to grow and to function. The instructions which enable these many intra-cellular reactions to take place, the genetic information, are encoded in the chromosomes, and we are interested in knowing how this information is stored, replicated and processed.

The secret lies in the chemical substance known as deoxyribonucleic acid, or DNA, for this is the genetic material and the most important constituent of the chromosomes (chapter 2). DNA is a very long polymer known as a *polynucleotide,* made up of four types of building block units or *nucleotides.* A molecule of DNA is many thousands of units long and it is the particular sequence of the nucleotide blocks along the molecule that encodes the genetic information. So important is the exact sequence of the blocks in a gene—which may be up to 1500 blocks long—that the replacement of one block by a different block (mutation) may completely destroy the normal activity of the gene. The structure of the four building blocks, the deoxyribonucleotides of adenine (A), thymine (T), guanine (G) and cytosine (C), is such that they occur in pairs, A with T and C with G, so that in fact the molecule of DNA consists of *two* chains of nucleotides paired off along their length (figure 1.4), and, because of the precise pairing relationships the sequence of nucleotides in one chain is determined by the sequence in the other chain; in other words the two chains are *complementary* to each other. The replication of such a molecule is easy; the two chains can separate and each separate chain can then act as a *template* for the formation of a new complementary chain. Because of the exact pairing each daughter molecule will have exactly the same nucleotide sequence as the parental molecule, so preserving unaltered the genetic information.

In some viruses the genetic material is not DNA but a closely related nucleic acid called ribonucleic acid or RNA. RNA is made up from the ribonucleotides of adenine, guanine and cytosine, uracil (U, which replaces and behaves as thymine in DNA) and it is usually single stranded rather than double stranded.

We must next ask, what is the function of a gene? Except for some genes which produce ribonucleic acid as their end product and others which are involved in control processes, the vast majority direct the synthesis of protein molecules; these proteins may be structural, contributing to the fabric of the cell, or, more usually, *enzymes* which enable the intricate biochemical processes of cell metabolism to take place (chapter 11).

Protein molecules are also long chain polymers, made up of twenty types of repeating units called *amino acids,* and are commonly 200–400 units long. The primary product of gene action is one of these long chains of amino acids, a polypeptide chain, which subsequently folds up according to a highly specific

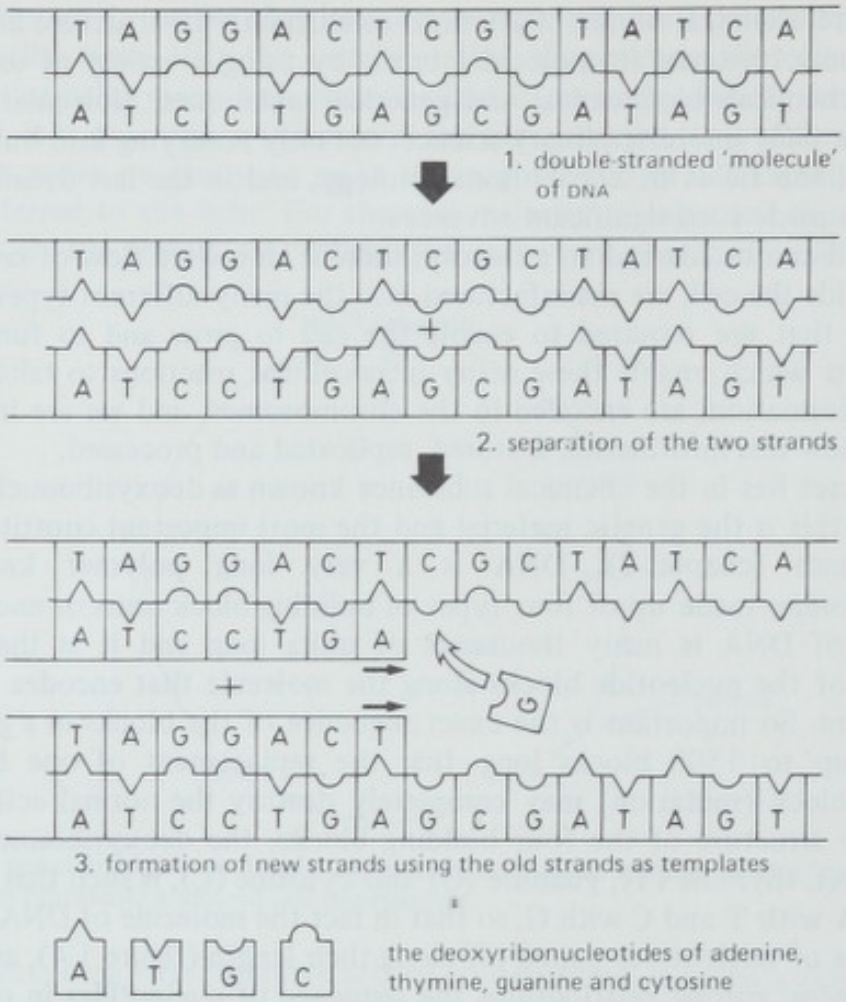


Figure 1.4 DNA replicates by a template mechanism. The nucleotides are represented by the four shapes, which can only fit together as two complementary pairs, A–T and C–G. The double-stranded molecule replicates by the strands separating and the two old strands acting as templates for two new strands. Both the daughter molecules are identical to the parent molecule.

pattern determined by the amino acid sequence. Many proteins consist of more than one such sub-unit—thus the haemoglobin molecule is an aggregate of four sub-units, two pairs of identical polypeptides. It is the exact shape of the protein molecule that determines its precise catalytic acitivity and this is, in turn, determined by the amino acid sequence. It should now be clear that the most important feature in protein synthesis is that the amino acids must be joined together in the correct order for each protein; somehow the four-letter code of the DNA has to be translated into the twenty-letter code of a protein. Furthermore, the proteins cannot be synthesised using DNA as a template since DNA is virtually confined to the nucleus whereas protein synthesis occurs along the ribosomes (figure 1.1).

The first key to protein synthesis is the existence of another specific nucleic acid, messenger ribonucleic acid, or mRNA, and the first step in protein synthesis is to copy, or *transcribe,* the nucleotide sequence of the gene on to a

molecule of mRNA. This is easy as the mRNA can be templated against the DNA and, because of the specific nucleotide pairing, will have the same nucleotide sequence as one strand and be complementary to the other strand of the DNA molecule. In effect, the nucleotide sequence of the double-stranded DNA molecule has been copied on to a single strand of mRNA (chapter 13). This mRNA now moves to the ribosomes and acts as a template on which the protein molecule is assembled (figure 1.5); this is called translation (chapter 14).

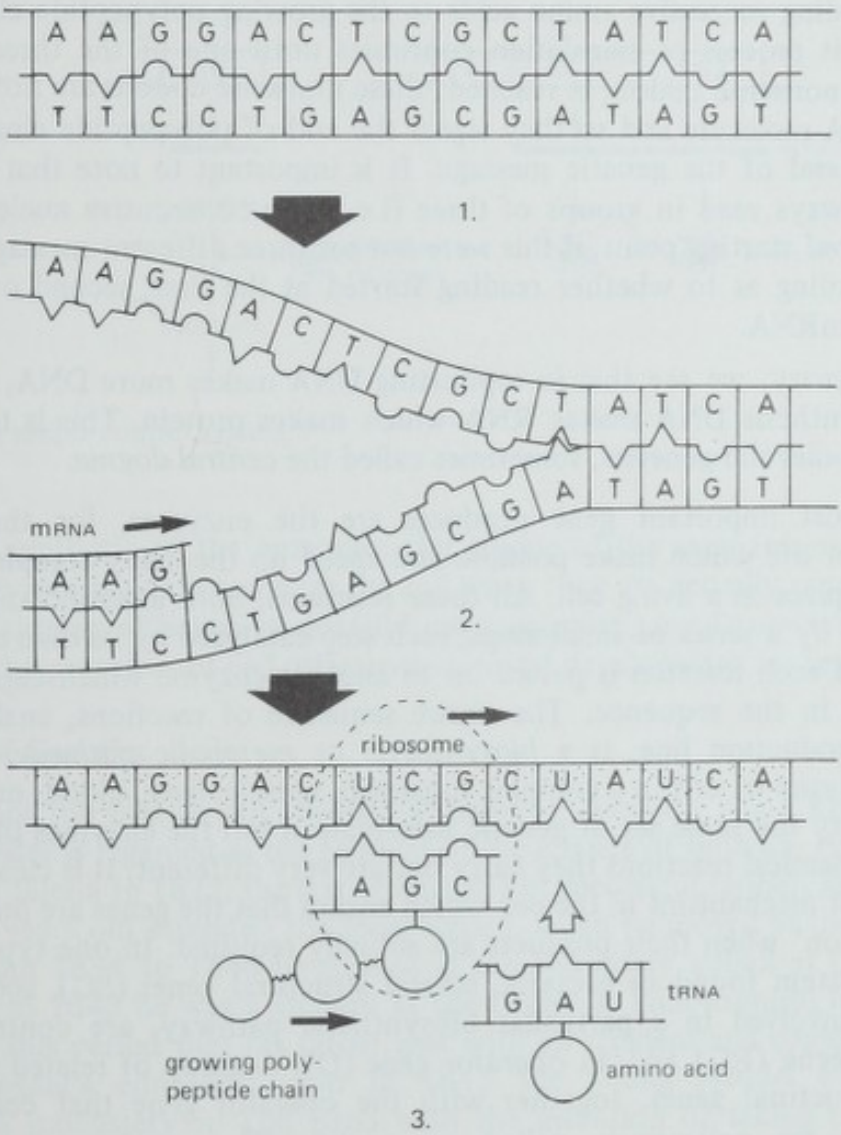


Figure 1.5 An outline of protein synthesis.

(1) Double-stranded molecule of DNA.

(2) A molecule of messenger RNA is templated against one of the strands of DNA. When completed, the molecule of mRNA passes to the ribosomes.

(3) A molecule of tRNA recognises the UCG codon on the molecule of mRNA and the amino acid it carries is joined on to the growing polypeptide chain. A molecule of a different tRNA is ready to attach to the next mRNA codon, CUA.

The second key is the existence of a specific transfer RNA, or tRNA, for each amino acid. Each tRNA is an adaptor molecule as it recognises on the one hand a particular amino acid, and on the other hand a particular sequence of three nucleotides, a *codon,* on the mRNA; a molecule of tRNA transfers the amino acid to the ribosomes, lines up on the mRNA template by recognising its own codon and the amino acid is joined on to the growing polypeptide chain. Altogether there are sixty-four possible codons, and sixty-one of these code for one or other of the amino acids, so that for some amino acids there are two or more different codons (chapter 12). The ribosomes move along the molecule of mRNA adding successive amino acids to the growing polypeptide chain as they do so. This process of translation continues until one of the three remaining codons, a nonsense codon, is reached; these nonsense codons are not recognised by a tRNA molecule and so they signal the end of polypeptide elongation and mark the end of the genetic message. It is important to note that the genetic code is always read in groups of three (i.e. three consecutive nucleotides) and from a *fixed* starting point; if this were not so, three different messages could be read according as to whether reading started at the first, second or third base along the mRNA.

In summary, we see that in replicating DNA makes more DNA, but that in protein synthesis DNA makes RNA which makes protein. This is the essential thesis of molecular genetics, sometimes called the *central dogma.*

The most important gene products are the enzymes, for these are the catalysts of life which make possible and speed up the many complex reactions that take place in a living cell. All these reactions, both anabolic and catabolic, take place by a series of small steps, each step catalysed by its own enzyme; the product of each reaction is passed on to another enzyme which carries out the next step in the sequence. The entire sequence of reactions, analogous to a factory production line, is a *biosynthetic* or *metabolic pathway.* The many different types of cell in a complex organism, muscle, liver, blood, nerves and so on, all carry the same set of genetic information but the enzymes they produce and the chemical reactions they carry out are very different. It is clear that there are control mechanisms in the cell which ensure that the genes are only active or 'switched on' when their products are actually required. In one type of simple control system found in bacteria, several structural genes (SG), specifying the enzymes involved in a particular biosynthetic pathway, are controlled by a *regulator* gene (RG) and an *operator* gene (O). A group of related and closely linked structural genes, together with the operator gene that controls their expression are collectively called an *operon.* The regulator gene specifies molecules of a specific repressor protein, which binds to the operator and prevents transcription (the production of mRNA) of the structural genes adjacent to the operator (figure 1.6a). However, when the substrate on which the enzymes act is present, molecules of the substrate complex with the repressor protein; this inactivates the repressor (figure 1.6b) so that it can no longer attach to the operator with the result that the structural genes can be transcribed and the enzymes synthesised (chapter 15). The substrate is said to

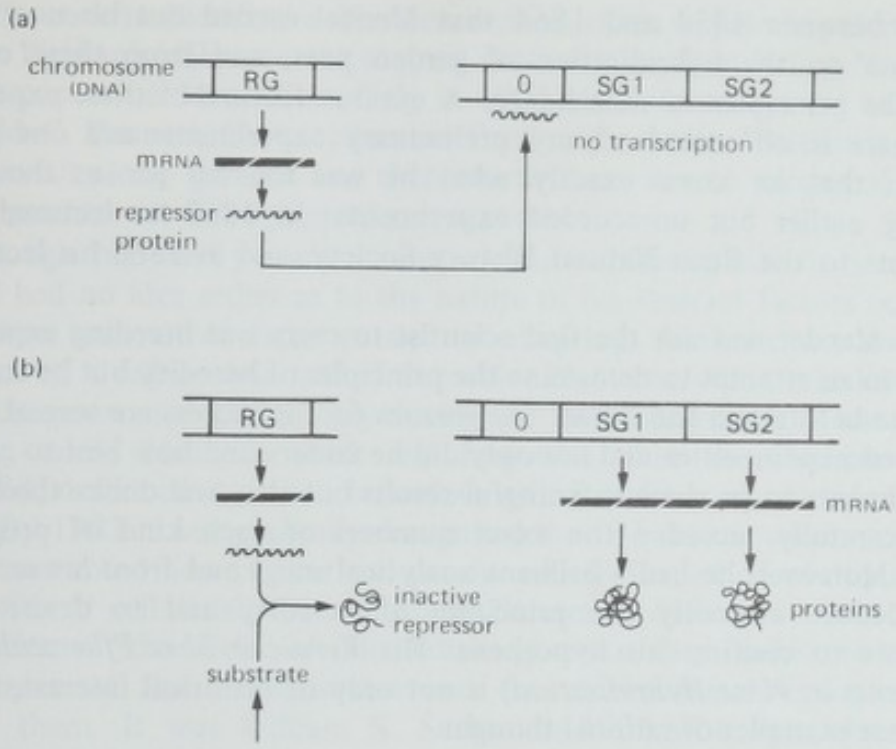


Figure 1.6 A simple control system.

induce the synthesis of the enzymes; this is one of the mechanisms to ensure that the enzymes are only made by the cell when they are actually required.

It is by similar, although necessarily more complex, regulatory systems that we believe the processes of cell differentiation and development are regulated.

The Birth of Genetics

Although not the first to put forward a theory of heredity, the Catholic priest Gregor Mendel is regarded as the founder of the science of genetics. He was born in Austrian Silesia in 1822, the son of a small but successful farmer, and until he was eleven years old attended the local school, when he moved to the school at Lipnik. From 1834 to 1840 he studied at the secondary school at Opava, and from 1840 to 1843 he was a student at the Olomouc Philosophical Institute. It was during this period that his father met with an accident and could no longer finance Mendel's education, and in 1843 the young Mendel entered the Augustinian monastery in Old Brno with the intention of taking part in the teaching work of the order. He was sent to study science at the University of Vienna so that he could qualify as a school teacher, but because of his lack of a formal education he twice failed his exams. Yet in spite of this he continued to participate in the teaching work of the order for much of the rest of his life. In addition to his ecclesiastical duties, Mendel had many other interests and was, for example, Vice-President of the Brno Mortgage Bank and Leader of the Brno Observatory. Mendel was ordained in 1847 and in 1868 was elected Abbot of the monastery; he died in 1884 as a result of a chronic infection of the kidneys.

It was between 1854 and 1864 that Mendel carried out his now famous experiments on the hybridisation of garden peas, and from these correctly deduced the principles of inheritance. A curious feature of these experiments is that there is no record of any preliminary experiments and one gets the impression that he knew exactly what he was looking for—as though just confirming earlier but unrecorded experiments. In 1865 he lectured on his experiments to the Brno Natural History Society, and in 1866 his lecture was published.

Gregor Mendel was not the first scientist to carry out breeding experiments with peas in an attempt to determine the principles of heredity but he succeeded while those before him had failed. The reasons for his success are several. Mendel was a gifted experimenter and not only did he understand how best to carry out the experiments to produce meaningful results but also, and unlike those before him, he carefully recorded the exact numbers of each kind of progeny he observed. Moreover, he had a brilliant analytical mind and from his results was able to deduce correctly the principles of heredity and to devise further experiments to confirm his hypothesis. His *Versuche über Pflanzenhybriden* (*Experiments in Plant Hybridisation*) is not only of historical interest, it is one of the finest examples of rational thought.

The importance of this work was that it recognised that it was not the characters themselves that were inherited, but determinants of characters. Earlier theories, as far back as Hippocrates and Aristotle, always envisaged that in some way it was the characters themselves that were inherited; Charles Darwin, for example, thought in 1868 that 'gemmules' from all parts of the body migrated into the germ cells, and eventually controlled the embryonic development of the organs from which they were derived.

Mendel's work now lay forgotten for thirty-five years, and then in 1900 it was independently rediscovered by Hugo de Vries, Carl Correns and Erich von Tschermak. To their disappointment they read of Mendel's discoveries just before publishing their own results and realised that they had been anticipated by nearly forty years. It is important to realise that this rediscovery was not simply a recognition of Mendel's work but a discovery of the principles of inheritance for the second time; thus even if Mendel had not made his discoveries it is unlikely that the history of genetics would have been greatly different.

The republication of Mendelian theory in 1900 caused a long and bitter controversy that was only to end with the death of one of the main antagonists, George Weldon. The chief protagonist of Mendelism was a Cambridge biologist, William Bateson, later to be the first Balfour Professor of Genetics at Cambridge. He read of the rediscovery of Mendelism on his way to London to lecture to the Royal Horticultural Society; realising its importance he hastily revised his lecture and so was the first to bring Mendelism to the British Isles. Weldon, Linacre Professor of Zoology at Oxford and joint founder of the journal *Biometrica*, was a firm supporter of Francis Galton's law of ancestral heredity which postulated that all variation was continuous and that there was a definite degree of relationship between the individuals in a family, and he continually belittled

Mendel's work in *Biometrica.* Bateson replied, often in private published papers because Weldon's influence was so great that no journal, including *Nature,* would publish papers on Mendelism. Only in 1904 did Bateson win his battle and, as Reginald C. Punnett, his successor at Cambridge, wrote, 'Bateson's generalship had won all along the line and thenceforth there was no danger of Mendelism being squashed out through apathy or ignorance'.

Although his theory went a long way to explaining the facts of heredity, Mendel had no idea either as to the nature of his abstract factors or as to how they were transmitted from parent to offspring. While he was aware of the existence of a cell nucleus and of threadlike filaments within it he knew nothing about chromosomes. It was only during the last two decades of the nineteenth century, while Mendel's work lay forgotten, that the chromosomes were identified, described and their behaviour at cell division recorded in the greatest detail. By 1903 it was generally recognised that the chromosomes occur in pairs and it was noted how regular and precise was their method of duplication and segregation at cell division; yet although Bateson and his co-workers made many discoveries in the field of Mendelian genetics they failed to realise the significance of the chromosomes and the chromosomal theory of heredity eluded them. It was William S. Sutton in 1903 who first appreciated the similarities between the behaviour of the chromosomes and the behaviour of Mendel's factors (or genes as we now call them, a term coined by Wilhelm Johannsen in 1909) and suggested that the chromosomes were the physical basis of the genetic material. He fully appreciated that the number of characters possessed by an individual greatly exceeds the number of chromosomes, so that each chromosome must carry a large number of genes. Unfortunately, Sutton was unable to prove his theory. At about the same time the great American school of *Drosophila* genetics led by Thomas Hunt Morgan and including Alfred Sturtevant and Calvin Bridges and later Hermann J. Muller was getting under way and by 1910 was able to confirm the chromosome theory in the most amazing detail. Within a very short time, what had been two separate subjects of research became united into the science of cytogenetics–the integration of cytological and genetical research.

The first forty years of this century saw the elucidation of the mechanism of inheritance in a wide range of organisms–*Drosophila,* maize, mouse and to a lesser extent man–and may be termed the era of classical cytogenetics. At the same time evolutionary theory moved from the nineteenth-century concept of blending inheritance to a foundation of Mendelian genetics, and the study of quantitative or continuous variation became part of the unified theory of Mendelian genetics.

Although we will describe some of these early discoveries in more detail, this book is primarily concerned with discoveries in the field of biochemical and molecular genetics. The field of biochemical genetics was opened up in 1941 when George Beadle and Edward Tatum isolated biochemical mutants of *Neurospora crassa* and proposed that each gene functions by specifying the production of a particular enzyme (chapter 11) while molecular genetics was born in 1953 when James Watson and Francis Crick finally elucidated the

structure of DNA (chapter 2). The advances in these fields are described in the chapters that follow.

2 The Material Basis of Heredity

If man will begin with certainties he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties.
Francis Bacon (1561-1626)

Although Mendel realised that it was not characters that were inherited but rather genetic factors, or genes as we call them, he had no idea as to their nature; it was only much later, after it had been firmly established that the genes were organised into chromosomes, that it became possible to isolate the genetic material from the rest of the cell and to attempt to elucidate its chemical nature and structure. Even so, between 1883 and 1889, August Weissmann, unaware of Mendel's results, developed his theory on the continuity of the germ plasm and not only proposed that the hereditary material was passed on intact through the germ line of one generation to the next, but also, and quite remarkably, suggested that the hereditary material was a particular chemical having a definite molecular structure.

Chemical analysis of plant and animal chromosomes has shown that they consist of two main components, proteins and nucleic acids; both of these are long chain molecules (polymers) made up of many similar but not necessarily identical subunits (monomers) held together by chemical bonds. A protein molecule (chapter 11) may be several hundred amino acid subunits long and as there are twenty different amino acids commonly found in proteins the number of possible permutations is astronomical; on the other hand most nucleic acids are made up from only four different nucleotides so that the number of possible permutations is very much smaller. At one time it also seemed that there was very little difference between the nucleic acids of one organism and another, and so the idea gradually came about that the active component of the chromosomes and genes was protein. It was not until 1944 that this was disproved and a nucleic acid, deoxyribonucleic acid or DNA, shown to be the genetic material.

The Genetic Material is DNA (or sometimes RNA)

Although nucleic acids were discovered by the Swiss biochemist Friedrich Miescher in 1897 their significance was not appreciated until 1944, when Oswald T. Avery, Colin M. MacLeod and Maclyn McCarty published the results of their experiments with the bacterium *Streptococcus pneumoniae*, or pneumococcus for short, and identified the genetic material as DNA. Their experiments were made possible by the discovery of transformation in pneumococci by Fred Griffith in 1928.

Transformation

Pneumococci are round bacteria which cause pneumonia in humans and septicaemia in mice. Many different strains are known but only the smooth (S) strains can cause the disease; these *virulent* strains have a gummy capsule of polysaccharide around each cell which protects them from destruction by the normal defence mechanisms of the host organism, and when grown on a synthetic medium in a Petri dish each bacterium grows into a smooth shiny colony. Other strains lack this capsule, do not cause disease symptoms and grow into rough (R) colonies. Griffith showed that when heat-killed S and living avirulent R bacteria were injected into mice not only did many of the mice die of septicaemia but *living* S type bacteria could be recovered from their heart blood (figure 2.1); neither the living R nor the dead S pneumococci caused septicaemia when injected separately. In some way the heat-killed S bacteria had *transformed* some of the R bacteria to the S type; there appeared to be a substance, or *transforming principle*, in the S cells which was capable of entering the R cells and there causing a stable heritable change.

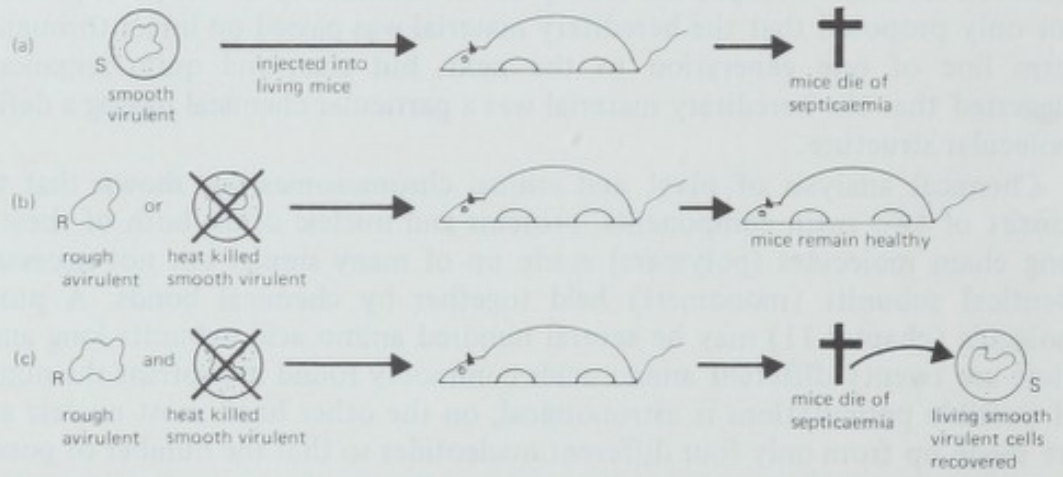


Figure 2.1 *In vivo* transformation. In 1928 Griffith showed that when smooth virulent (S) pneumococci are injected into mice the mice die of septicaemia (a), but that when either rough avirulent (R) or heat-killed smooth virulent bacteria are injected the mice remain healthy (b). When living rough *and* heat-killed smooth bacteria were injected together the mice died of septicaemia and living smooth virulent bacteria were recovered from their heart blood (c).

Avery and his co-workers, after ten years of work, succeeded in carrying out this process of transformation *in vitro* ('in the test tube') instead of *in vivo* ('in living cells'). They extracted the DNA, protein and capsular components from living S pneumococci and mixed each component with living R type bacteria suspended in a synthetic medium, and they found that the DNA fraction, and only the DNA fraction, could convert some of the R cells to the S type. Furthermore, the higher the purity of the DNA the more efficient was this process of transformation, while if the DNA was first treated with the enzyme deoxyribonuclease (DNase), which causes the breakdown of DNA, no transformation occurred (figure 2.2). Other enzymes had no effect on the

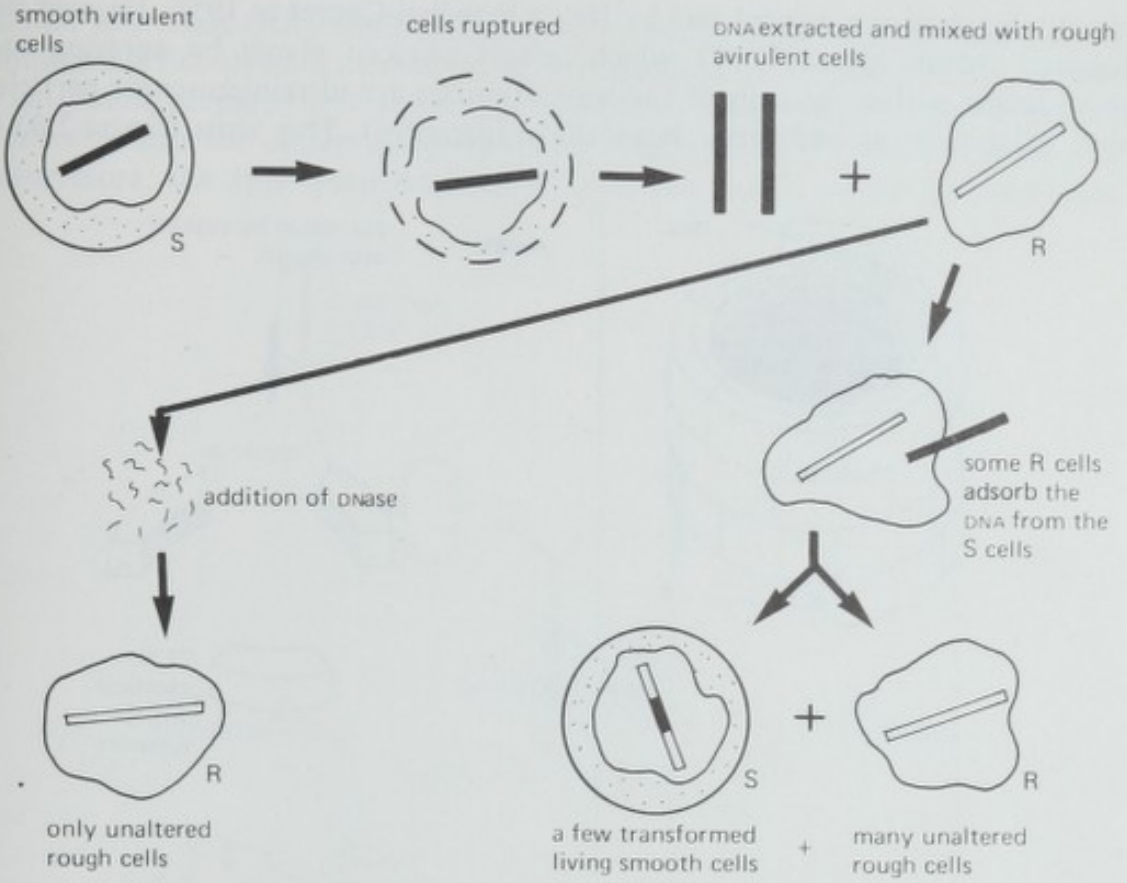


Figure 2.2 *In vitro* transformation. When DNA extracted from a smooth strain of pneumococci is mixed with living rough bacteria, a few of the rough bacteria are transformed to smooth. This transformation is prevented by the addition of DNase.

transforming ability of the extract. Hence, when DNA from cells of one genotype is incorporated into cells of a different genotype, stable heritable changes may be brought about; the DNA is endowed with specific genetic activity and is the genetic material.

Retrospectively, we accept without question this evidence that DNA is the genetic material, but in 1944 it was received with a certain scepticism. In spite of the demonstration that DNase destroyed the transforming activity, the critics continued to argue that transformation was the result of protein impurities in the DNA and that these were the active principle. Rollin D. Hotchkiss soon showed this to be unlikely by continuing to purify the DNA, until by 1949 the protein contamination had been reduced to a mere 0.02 per cent; not only did these very pure DNA preparations still effect transformation but the greater the purity of the DNA the higher was the frequency of transformation.

Note that transformation is a direct demonstration that it is not the character itself (in this case a polysaccharide capsule) but a genetic determinant, now identified as DNA, that is inherited.

Reconstituting a Virus

Another very convincing experiment demonstrating that nucleic acids are the

genetic material was carried out by Heinz Fraenkel-Conrat in 1956. He used the tobacco mosaic virus (TMV) which infects tobacco plants by entering and reproducing within the cells of the leaves (viruses are ultramicroscopic particles that exist only as obligatory intracellular parasites). This virus (figure 2.3) is

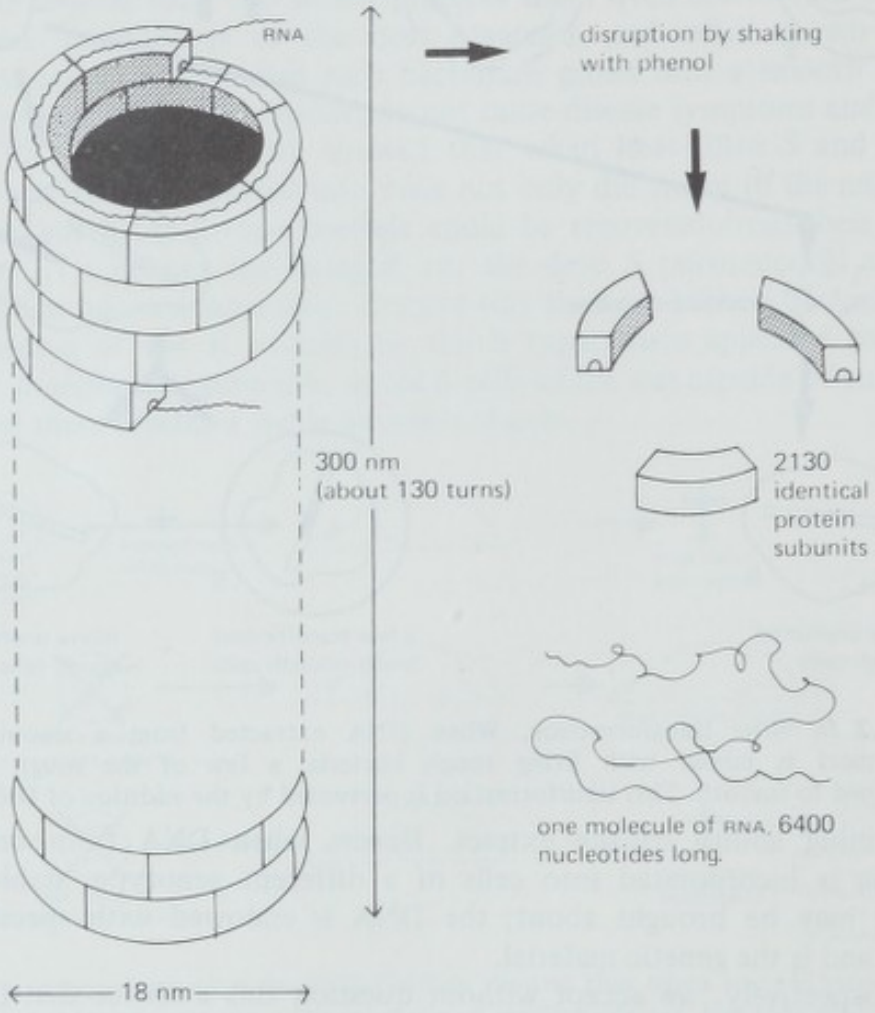


Figure 2.3 A schematic drawing of the structure of the tobacco mosaic virus. TMV (left) is a hollow rod made up of 2130 identical protein subunits with a molecule of single-stranded RNA wound helically between the subunits. When the intact virus is shaken with phenol it disrupts into the protein and RNA components (right).

composed of a large number of identical protein subunits arranged helically so as to form a hollow cylinder, and enclosing a molecule of RNA in the form of a flat spiral (in some viruses RNA, ribonucleic acid, is found instead of DNA). In TMV the protein and the RNA can be separated by chemical fractionation and the isolated RNA, but not the protein, can infect a tobacco plant and give rise to normal viral progeny; although this infectivity is rather inefficient, probably because the naked RNA is degraded by enzymes during the course of infection, it is completely destroyed by treatment with RNase. By using this method of separation and aggregation Fraenkel-Conrat was able to produce hybrid virus particles having the protein coat of the common TMV strain and the RNA of the

Holmes Rib Grass (HR) strain (figure 2.4). These hybrids, because they had the common TMV coats were inactivated by antibodies prepared against TMV but not by antibodies prepared against the HR strain; when these hybrid viruses were used to infect plants the disease symptoms were always those of the RNA donor and, furthermore, the virus particles isolated from the lesions on the infected plant

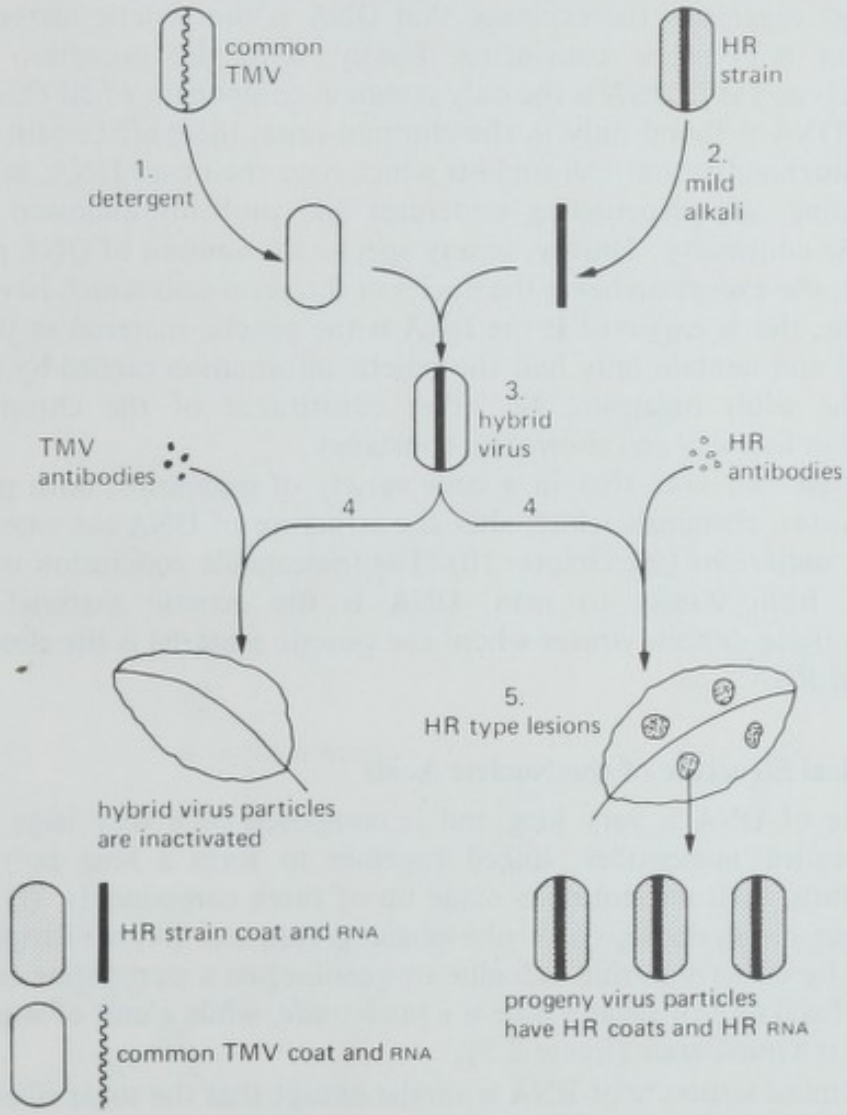


Figure 2.4 Reconstituting a virus.
(1) Common TMV coats are prepared by treating TMV particles with detergent solution.
(2) RNA is prepared from the Holmes Rib Grass (HR) strain by treatment with mild alkali.
(3) The common TMV coats and the HR RNA are re-aggregated to form hybrid virus particles.
(4) These hybrids are inactivated by antibodies prepared against the common TMV strain but not by antibodies prepared against the HR strain.
(5) When the hybrid particles infect a plant the lesions are characteristic of those of the HR strain.
(6) The virus particles isolated from these lesions have both HR RNA and HR coats and so are inactivated by the antibodies prepared against the HR strain.

were inactivated by the antibodies prepared against the HR strain. Quite clearly these second generation virus particles have both the RNA and the protein coats of the HR strain; it is the RNA, and not the protein, that contains all the genetic information necessary for the replication and production of new virus particles.

The experiment described on page 45, the *in vitro* synthesis of a bacterial virus, is also a direct demonstration that nucleic acids are the genetic material.

In higher organisms the evidence that DNA is the genetic material is more indirect but nonetheless convincing. Firstly, with the exception of certain viruses, such as TMV, DNA is the only common component of all chromosomes. Secondly, DNA is found only in the chromosomes; there are certain exceptions such as mitochondria and chloroplasts which have their own DNA, but these are self-replicating, self-perpetuating structures and probably endowed with their own genetic continuity. Thirdly, in any species the amount of DNA per nucleus is constant, the exception being the nuclei of the germ cells which have only half this amount; this is expected if the DNA is the genetic material as the gametes are haploid and contain only half the genetic information carried by the diploid cells of the adult organism. No other constituent of the chromosomes is distributed in this way and shows this constancy.

Finally, we can note that in a wide variety of organisms, both procaryotes and eucaryotes, chemicals which alter the structure of DNA can cause heritable changes or *mutations* (see chapter 10). The inescapable conclusion is that in all organisms, from viruses to man, DNA is the genetic material—the only exceptions being certain viruses where the genetic material is the closely related nucleic acid RNA.

The Chemical Structure of the Nucleic Acids

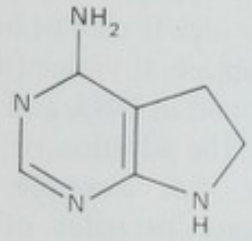
A molecule of DNA is very long and is composed of a very large number of subunits, called nucleotides, linked together to form a long *polynucleotide chain.* In turn, each nucleotide is made up of three components: (i) a molecule of the sugar deoxyribose, (ii) a phosphate group, and (iii) a nitrogenous base, which can be either a purine (adenine or guanine) or a pyrimidine (cytosine or thymine). Each unit of sugar + base is a *nucleoside,* while a unit of sugar + base + phosphate is a *nucleotide* (figure 2.5).

The chemical structure of RNA is similar except that the sugar ribose is found instead of deoxyribose, and the pyrimidine uracil replaces thymine.

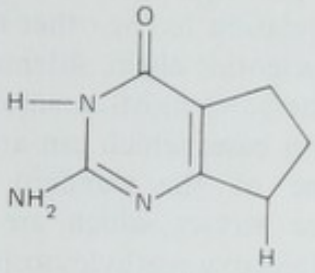
The nucleotide monomers are joined together by their phosphate residues, each of which links together the 3′ carbon atom of one deoxyribose molecule and the 5′ carbon of the next deoxyribose molecule by a 3′:5′ phospho-diester linkage (figure 2.6). One very important point is that because of this asymmetry the DNA molecule is *polarised.* If the molecule starts at one end with a carbon in

Figure 2.5 The components of DNA and RNA. The basic components of DNA are the four nitrogenous bases (adenine, guanine, cytosine and thymine), phosphoric acid and the sugar 2′deoxy-d-ribose. In RNA uracil replaces thymine and d-ribose replaces 2′deoxy-d-ribose. The combination of sugar + base is a nucleoside, converted to a nucleotide by the addition of one or more phosphate groups.

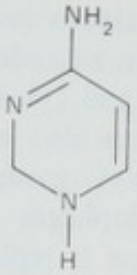
BASES



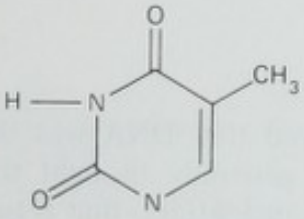
adenine



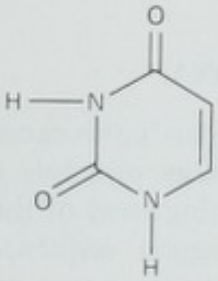
guanine



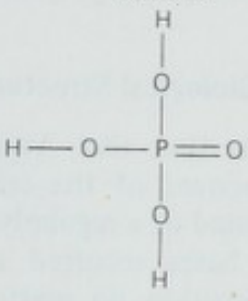
cytosine



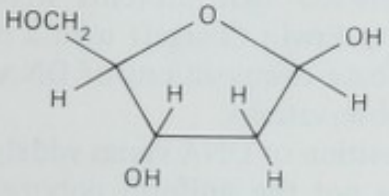
thymine



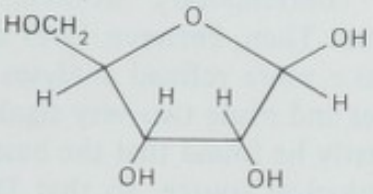
uracil



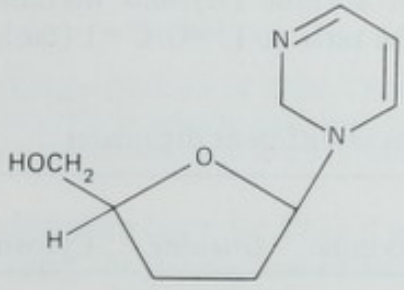
phosphoric acid



2′ deoxy-d-ribose

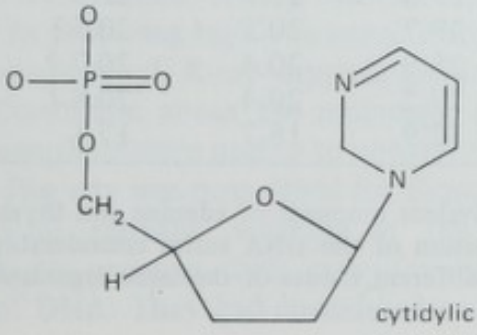


d-ribose



cytidine

NUCLEOSIDES
(sugar + base)
(or thymidine
adenosine
guanosine)



cytidylic acid

NUCLEOTIDES
(sugar + base + phosphate)
(or thymidylic acid
adenylic acid
guanylic acid)

the 5′ position (the top of the molecule in figure 2.6) then the other end will have a carbon in the 3′ position. A consequence of this polarity is that if a nucleotide is inverted in relation to the other nucleotides it cannot be attached onto an extending polynucleotide chain. Adenine, guanine, thymine (or uracil in RNA) and cytosine are the bases most commonly found in DNA and RNA, but there are also less common bases which can arise by the addition of methyl or hydroxyl groups to one of the standard bases. For example, in some bacteriophages (these are viruses which are obligate parasites of bacteria) cytosine is replaced by hydroxy-methylcytosine (HMC), while in some higher organisms methylcytosine is found.

The Biological Structure of DNA

At the time that Avery and his co-workers showed that DNA was the active component of the transforming principle it was generally thought that DNA consisted of a regularly repeating array of the four nucleotides; that is to say, the four bases recurred in a regular sequence so that DNA was of the same composition no matter what its source and so incapable of carrying genetic specificity (this was the tetranucleotide theory). Even after Avery's experiments many contemporary workers still believed that proteins were the genetic material. Then, between 1949 and 1951, Erwin Chargaff used a new technique to make more refined analyses of the base compositions of DNA from various sources and made two very significant observations.

Firstly he found that the base composition of DNA varies widely according to its biological source, so that DNA was not the uniform polymer as had been widely believed up to then and it could therefore carry the genetic information.

Secondly he found that there were always equimolar amounts (i.e. the equivalent numbers of molecules) of adenine (A) and thymine (T) and of cytosine (C) and guanine (G) so that the ratio A/T = G/C = 1 (table 2.1); we will

Table 2.1 Base ratios in different organisms

	Adenine	Thymine	Guanine	Cytosine	$\frac{A+T}{G+C}$
Escherichia coli	25.4	24.8	24.1	25.7	1.01
Wheat	26.8	28.0	23.2	22.7	1.21
Mouse	29.7	25.6	21.9	22.8	1.21
Pig: liver	29.4	29.7	20.5	20.5	
thymus	30.0	28.9	20.4	20.7	1.43
spleen	29.6	29.2	20.4	20.8	
Yeast	31.3	32.9	18.7	17.1	1.79

Note that although there are always equivalent amounts of adenine and thymine, and of guanine and cytosine, the overall composition of the DNA varies considerably from one organism to another, whereas DNA from different tissues of the same organism always has the same composition.

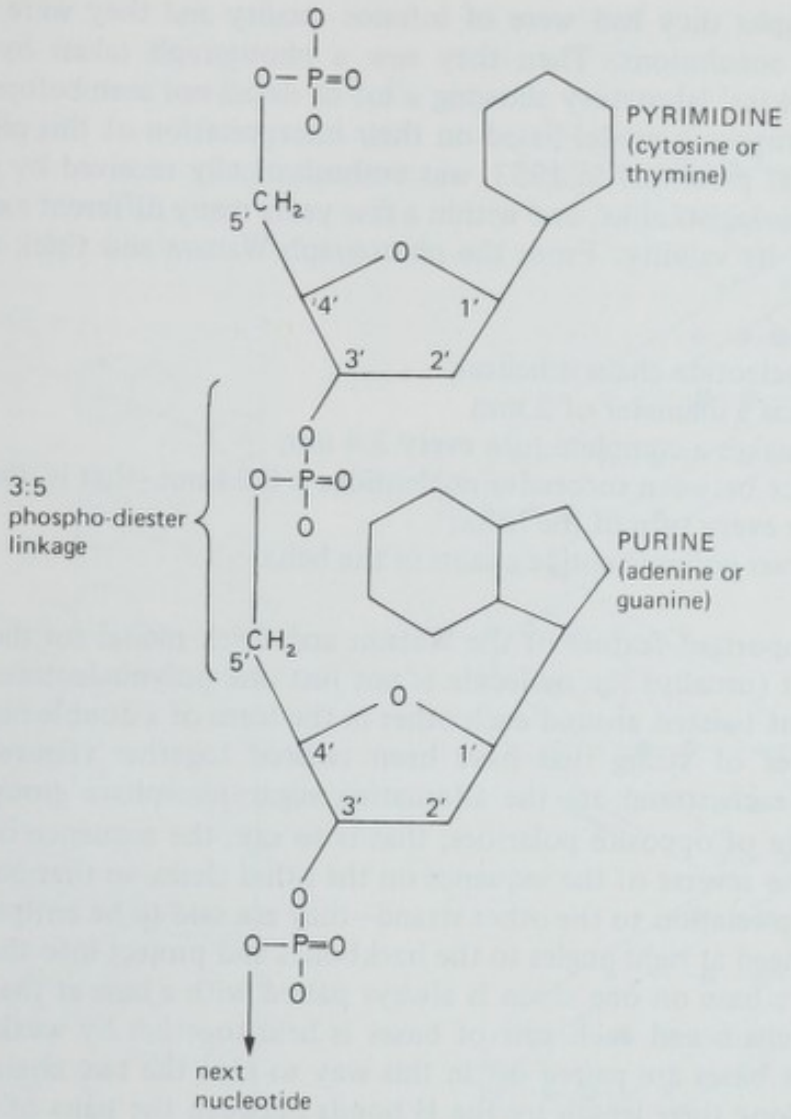


Figure 2.6 The primary structure of DNA. The backbone of DNA is made up of alternating phosphate and deoxyribose groups held together by 3:5 phospho-diester linkages. One of the four nucleotide bases is attached to the 1′ carbon of each deoxyribose.

return to this equivalent later. By 1952 it was universally accepted that the DNA molecule does carry the genetic information and that this information is stored in the specific sequences of the four nucleotide bases along the polynucleotide chains, and several teams were trying to elucidate its molecular structure.

One of these teams, led by Maurice Wilkins at King's College, London, had succeeded in preparing highly oriented fibres of DNA which they then examined by the technique of X-ray crystallography; the resulting diffraction patterns yielded information about the molecular structure of the crystal, but Wilkins and his co-workers were unable to convert their data into a reasonable molecular structure. The way was now paved for Francis Crick and James Watson, a visiting scientist from Harvard, working at the Medical Research Laboratories in Cambridge, to propose their now famous model for the three-dimensional structure of DNA. They had considered a number of possible structures, but the

X-ray photographs they had were of inferior quality and they were unable to come to any conclusions. Then they saw a photograph taken by Rosalind Franklin in Wilkins' laboratory showing a lot of detail not seen before and they were able to propose a model based on their interpretation of this photograph. This model, first presented in 1953, was enthusiastically received by physicists, chemists and biologists alike, and within a few years many different experiments had confirmed its validity. From the photograph Watson and Crick concluded that

(1) the polynucleotide chain is helical;
(2) the helix has a diameter of 2 nm;
(3) the helix makes a complete turn every 3.4 nm;
(4) the distance between successive nucleotides is 0.34 nm—that is, there are ten nucleotides for every turn of the helix;
(5) there are *two* polynucleotide chains in the helix.

The first important feature of the Watson and Crick model for the structure of DNA is that (usually) the molecule is not just one polynucleotide chain but *two* such chains twisted around each other in the form of a double helix—rather like two pieces of string that have been twisted together (figure 2.8). The backbones of each strand are the alternating sugar-phosphate groups and the two strands are of opposite polarities; that is to say, the sequence of atoms in one chain is the reverse of the sequence on the other chain, so that one strand is upside down in relation to the other strand—they are said to be antiparallel. The bases are arranged at right angles to the backbones and project into the centre of the molecule; a base on one chain is always paired with a base at the same level on the other chain and each pair of bases is held together by weak hydrogen bonds. All the bases are paired off in this way so that the two chains are held together all along their length by the H bonds between the pairs of bases. The whole structure is analogous to a rope ladder twisted along its axis, where the side ropes correspond to the sugar-phosphate backbones, and the rungs to the paired bases. The two and three-dimensional structures of DNA are shown in figures 2.7 and 2.8.

The second important feature is that the pairing of the bases is not random. If the molecule is to be regular and undistorted then its dimensions are such that there is not room to accomodate a pair of the large purine bases (A and G) while a pair of pyrimidines (C and T) is too small. Furthermore, a base with two hydrogen bonds (A or T) cannot normally pair with a base with three bonds (C or G), so that the only possible pairs are adenine with thymine and cytosine with guanine. As a consequence one chain is always complementary to the other chain; if there is a sequence A A T C G A T C on one chain then the corresponding sequence on the other chain will be T T A G C T A G. Therefore it is a requirement of this model that there be equimolar amounts of adenine and thymine and of cytosine and guanine, exactly as was observed by Chargaff (table 2.1). Observe that the model places no restriction on the order of the base pairs along the molecule so that there is no reason why the ratio of the numbers

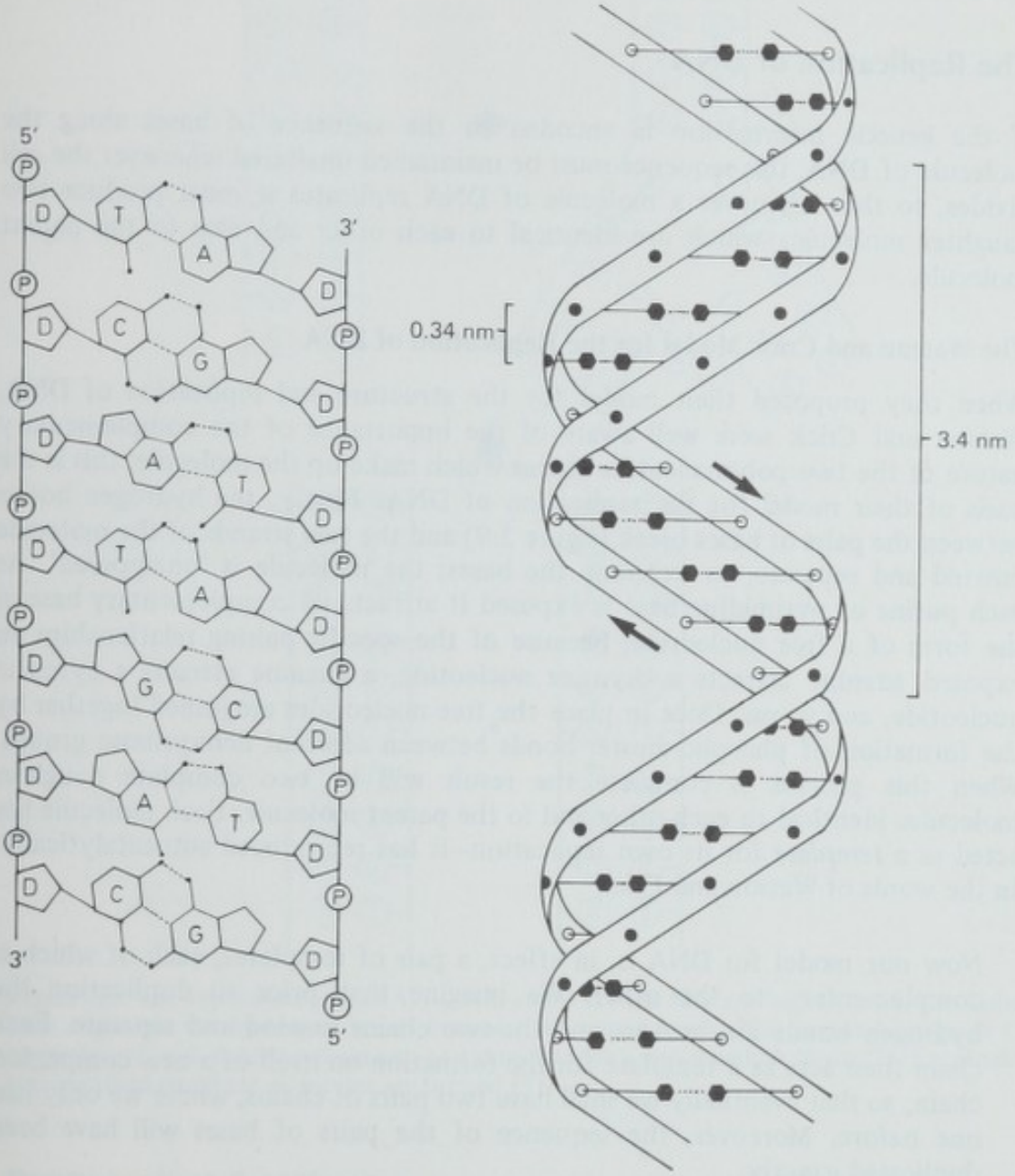


Figure 2.7 The two-dimensional structure of DNA. The molecule consists of two polynucleotide chains, polarised in opposite directions, with pairs of complementary bases held together by weak hydrogen bonds (represented by the dashed lines). D: deoxyribose; A: adenine; T: thymine; P: phosphate; G: guanine; C: cytosine.

Figure 2.8 The structure of DNA. The pair of 'ribbons' represents the sugar–phosphate backbone chains. The bases (hexagons) are held together by weak hydrogen bonds (dashed lines) and attached to the deoxyribose groups (circles).

of A-T to G-C pairs should be the same from one organism to another; again, this is consistent with Chargaff's data.

The Replication of DNA

If the genetic information is encoded in the sequence of bases along the molecule of DNA, this sequence must be maintained unaltered whenever the cell divides, so that whenever a molecule of DNA replicates it must produce two daughter molecules which are identical to each other and also to the parent molecule.

The Watson and Crick Model for the Replication of DNA

When they proposed their model for the structure and replication of DNA, Watson and Crick were well aware of the importance of the complementary nature of the two polynucleotide chains which make up the molecule; this is the basis of their model for the replication of DNA. Firstly, the hydrogen bonds between the pairs of bases break (figure 2.9) and the two strands of the molecule unwind and separate, so exposing the bases; the molecule is 'unzippered'. As each purine or pyrimidine base is exposed it attracts its complementary base in the form of a free nucleotide; because of the specific pairing relationships an exposed adenine attracts a thymine nucleotide, a guanine attracts a cytosine nucleotide, and so on. Once in place the free nucleotides are joined together by the formation of phospho-diester bonds between adjacent deoxyribose groups. When this process is complete the result will be two complete daughter molecules identical to each other and to the parent molecule. Each molecule has acted as a *template* for its own replication—it has reproduced autocatalytically. In the words of Watson and Crick

> Now our model for DNA is, in effect, a pair of templates, each of which is complementary to the other. We imagine that prior to duplication the hydrogen bonds are broken, and the two chains unwind and separate. Each chain then acts as a template for the formation on itself of a new companion chain, so that eventually we shall have two pairs of chains, where we only had one before. Moreover, the sequence of the pairs of bases will have been duplicated exactly.

This type of replication is termed *semi-conservative,* because although the two original strands are conserved they are separated, one into each of the daughter molecules, and serve as templates for the formation of new strands.

Alternative models would be for an intact double helix of DNA to act as a template for a new double helix, so that the parental molecule is retained intact (conservative replication), or for the parental molecule to break down and for the fragments to be distributed among the two daughter molecules (dispersive replication).

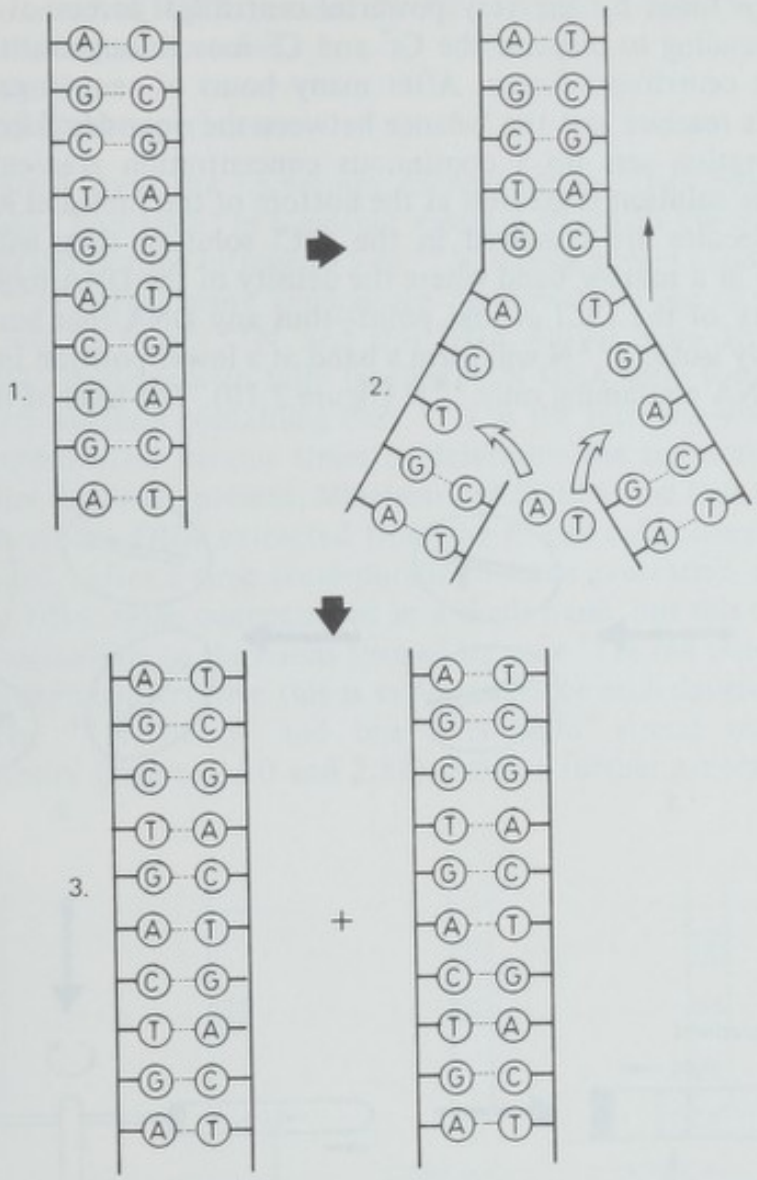


Figure 2.9 The replication of DNA. A, T, C and G are the four nucleotide bases, the dashed lines represent the hydrogen bonds between the paired bases. The parental molecule (1) unwinds and each separate chain acts as a template for a new daughter chain (2). Eventually, two identical daughter molecules are formed (3).

Density-gradient Centrifugation

Although Watson and Crick proposed their scheme for the replication of DNA in 1953, it was not until 1958 that Matthew Meselson and Franklin W. Stahl devised an experimental method for demonstrating that DNA does replicate semi-conservatively. This was made possible by using the then new technique of *density-gradient* centrifugation developed by Jerome Vinograd, which enables the separation of molecules with very small differences in their densities. When strong solutions of certain salts, such as caesium chloride, are subjected to very high centrifugal forces (100 000 *g*) the salt atoms are drawn to the bottom of

the centrifuge tubes by the very powerful centrifugal forces; at the same time diffusion is tending to disperse the Cs^+ and Cl^- ions throughout the solution, so opposing the centrifugal forces. After many hours of centrifugation a state of equilibrium is reached and the balance between the opposing forces of diffusion and sedimentation sets up a continuous concentration gradient of CsCl; the density of the solution is greatest at the bottom of the tube and least at the top. If DNA molecules are dissolved in the CsCl solution they will gradually be concentrated in a narrow band where the density of the DNA molecules is equal to the density of the CsCl at that point–thus any DNA that has been labelled with the heavy isotope ^{15}N will form a band at a lower position in the tube than will 'light' DNA containing only ^{14}N (figure 2.10). The level of the DNA in the

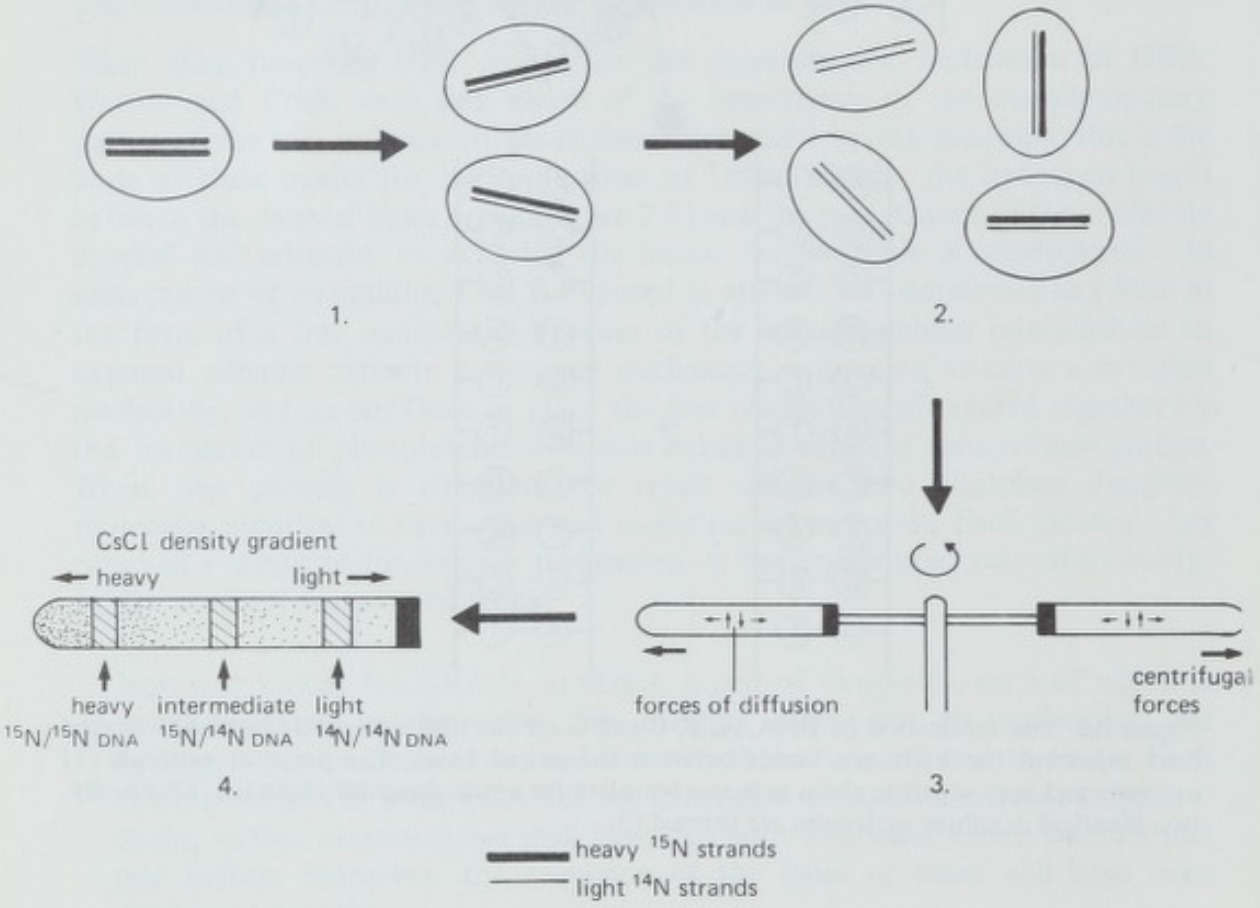


Figure 2.10 The Meselson and Stahl experiment.

(1) *E. coli* grown in ^{15}N medium so as to label the DNA 'heavy' in both strands.

(2) The cells are transferred to ^{14}N medium and allowed to replicate for up to four generations. All the new DNA strands are labelled 'light' with ^{14}N.

(3) At time intervals up to four hours the DNA was extracted, dissolved in CsCl and centrifuged.

(4) After many hours at 100 000 *g*, equilibrium is reached. The DNA concentrates in bands, each band having the same density as the CsCl gradient at that point. The position and concentration of the DNA are determined photographically.

tubes and the amount of DNA present can be determined from uv absorption photographs of the centrifuge tubes.

The Meselson and Stahl experiment

Meselson and Stahl grew the bacterium *Escherichia coli* (so called after its discoverer Theodor Escherich) in a culture medium in which the only nitrogen source was the heavy isotope nitrogen-15 or ^{15}N, having a mass greater than that of the normal ^{14}N. After many generations of growth all the nitrogen in the bacterial DNA will be heavy ^{15}N, and this DNA will be more dense than the normal ^{14}N DNA. Bacteria with fully labelled ^{15}N DNA were then transferred to normal culture medium containing only ^{14}N as the nitrogen source and the culture was sampled after various times to determine the amounts of DNA of different densities that were present. Meselson and Stahl found that after growth in ^{15}N medium all the DNA extracted from the *E. coli* cells concentrated in a single 'heavy' band. After a time corresponding to one generation cycle in ^{14}N medium, all the DNA again concentrated in a single band, but this band formed at a position intermediate to the bands formed by pure ^{15}N and pure ^{14}N DNA. If replication is semi-conservative this is expected, since each daughter molecule will contain one ^{15}N 'heavy' and one ^{14}N 'light' strand and so be of intermediate density (figures 2.10 and 2.11). After a further generation in ^{14}N

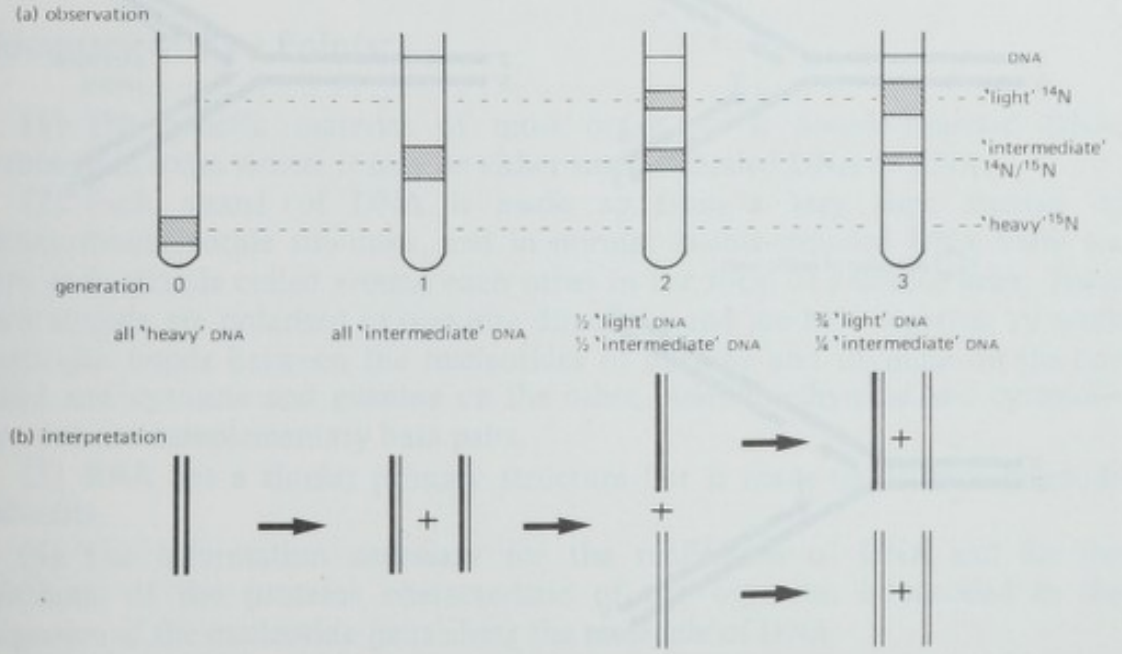


Figure 2.11 Observations and interpretation of the Meselson and Stahl experiment. The simplest explanation of the observed results is that each molecule of DNA replicates semi-conservatively, so that after one generation in ^{14}N medium all the daughter molecules have one 'heavy' ^{15}N and one 'light' ^{14}N strand. After a further generation in ^{14}N medium each of these hybrid molecules replicates to produce one $^{14}N/^{15}N$ hybrid molecule and one $^{14}N/^{14}N$ 'light' molecule, and so on. The ^{15}N labelled strands of each DNA duplex are represented by the thick lines and the ^{14}N unlabelled strands by the thin lines.

medium they observed one half of the DNA to be of intermediate density and one half to be 'light' DNA, again as predicted according to a semi-conservative method of replication. In each successive sample the distribution of the DNA was precisely that expected. The demonstration was made even more convincing when they gently heated the 'hybrid' $^{15}N/^{14}N$ DNA to separate the strands (this is called thermal denaturation) and recentrifuged it; they now observed two bands, one 'heavy' and one 'light', a convincing demonstration that the first generation hybrid molecules are two-stranded, containing one parental (^{15}N) and one new (^{14}N) strand.

Discontinuous Replication

We are still curiously ignorant as to the events that take place at the point of replication, the replication fork. There are two particular problems. One is that the only known enzymes that join together successive nucleotides in replicating DNA, known as *DNA-dependent DNA polymerases* (usually abbreviated to DNA polymerases) can operate *only* in the 5′ to 3′ direction; thus while DNA polymerase could replicate one strand of the duplex strictly according to the

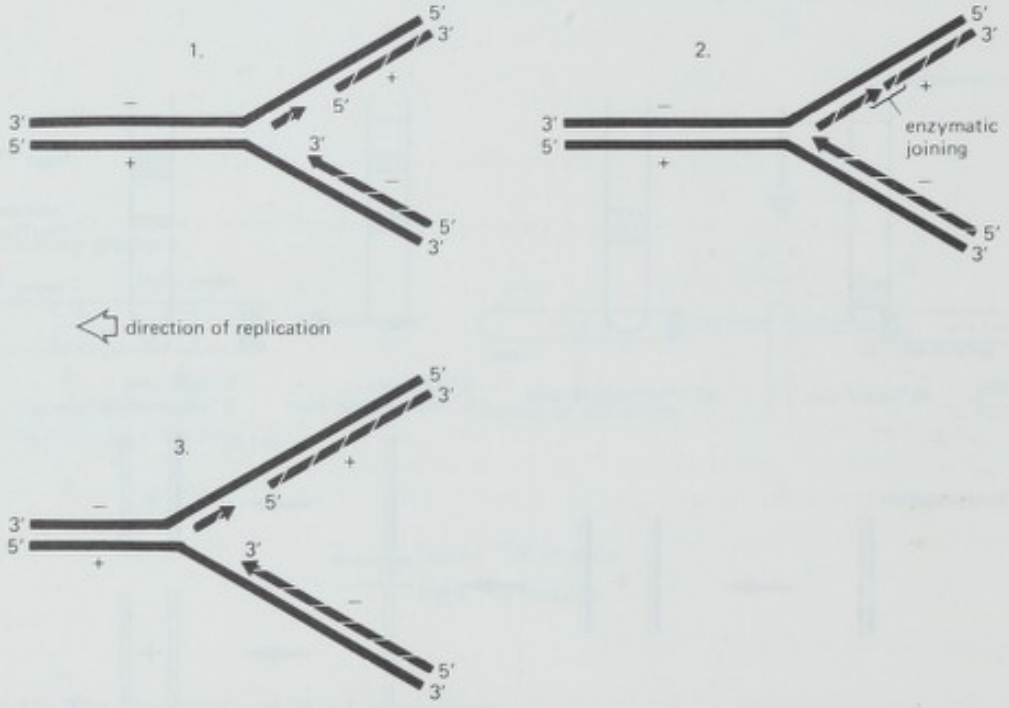


Figure 2.12 The discontinuous replication of DNA. The two strands of the double helix have partly separated and each is acting as a template for a new daughter strand. The parental (+) strand acts as a template for a continuous new (−) strand synthesised in the 5′ to 3′ direction. The new (+) strand is synthesised against the parental (−) strand as follows:
(1) a short fragment templates against the (−) strand and grows in the 5′ to 3′ direction;
(2) when completed this fragment is enzymatically joined on to the new (+) strand;
(3) meanwhile the double helix has further unwound and another fragment is being synthesised, also in the 5′ to 3′ direction.

model of Watson and Crick it could not replicate the other strand in the same way, since this would involve synthesis in the 3′ to 5′ direction. Exactly how the other strand replicates is not yet clear, but it seems that short pieces of DNA are templated in the 5′ to 3′ direction and then enzymatically joined on to the growing chain, so that actual synthesis is in the 5′ to 3′ direction even though the overall extension of this strand is in the 3′ to 5′ direction (figure 2.12). This idea of *discontinuous replication* immediately identified the need for an enzyme which could join together the completed sections of the new DNA chains and it was not long before such an enzyme was isolated; known as DNA ligase, this enzyme is able to join together the free ends of single strand breaks in the DNA duplex. The second problem, not yet fully understood, is that DNA polymerase not only requires a template strand to copy but also it can only add on deoxyribonucleotides to an existing nucleotide chain; thus each new section of DNA appears to need a *primer* to initiate DNA synthesis. We believe that each new section of DNA is primed by a short piece of RNA, synthesised by a DNA-dependent RNA polymerase, providing a short ribonucleotide sequence to which DNA polymerase can then add on deoxyribonucleotides. Presumably, these short pieces of RNA primer are enzymatically cleaved from the growing DNA chains before they are joined together by DNA ligase.

Summary of Key Points

(1) The genetic material of most organisms is double-stranded DNA, although in some viruses it may be either single-stranded DNA or RNA.

(2) Each strand of DNA is made up from a very large number of deoxyribonucleotide sub-units, and in normal double-stranded DNA there are two such strands coiled around each other in the form of a double helix. These two strands are polarised in opposite directions and are held together by weak hydrogen bonds between the nucleotides of adenine and thymine on the one hand and cytosine and guanine on the other. Adenine–thymine and cytosine–guanine are complementary base pairs.

(3) RNA has a similar primary structure but is made up of ribonucleotide subunits.

(4) The information necessary for the replication of DNA and for the synthesis of the proteins characteristic of the organism is encoded in the sequence of the nucleotide pairs along the molecule of DNA.

(5) When DNA replicates, the daughter molecules *must* contain the same sequences of nucleotides as the parent molecule. This is achieved by the two strands separating and each acting as a template for the formation of a complementary daughter strand. Thus each daughter molecule consists of one new and one old strand; this is termed semi-conservative replication.

(6) Special techniques described are transformation and density-gradient centrifugation.

3 The Chromosomes of Bacteria and Viruses

I drink for the thirst to come.
Appetite comes with eating.
François Rabelais (*c.* 1492–1553)

In higher animals and plants (eucaryotes) the chromosome is a highly complex structure (chapter 4) consisting of both DNA and protein and at certain times visible under the light microscope; it is of an entirely different order of magnitude to a molecule of DNA. In viruses and bacteria (procaryotes) the organisation of the chromosome is at its simplest possible level as it consists of a single molecule of DNA (bacteria, most bacteriophages and most animal viruses) or RNA (plant viruses, some animal viruses and some bacteriophages), sometimes linear and sometimes a covalently bonded circular molecule; these chromosomes are so simple that some geneticists prefer to call them *chromonemes* to distinguish them from the more complex chromosomes of eucaryotes. Except in bacteria containing more than one chromatin body, there is usually only one chromosome in each procaryotic cell. The physical characteristics of some procaryotic chromosomes are set out in table 3.1.

Table 3.1 The physical characteristics of some procaryotic chromosomes

Organism	M.W.	Type	Shape	Length μm	Nucleotides
E. coli	2×10^9	DNA[d]	circular	1100	3×10^6 pairs
T2 phage	1.3×10^8	DNA[d]	linear	56	2×10^5 pairs
λ phage	3.2×10^7	DNA[d]	linear/circular	16	5×10^4 pairs
X174 phage	1.7×10^6	DNA[s]	circular	1.7	5×10^3
TMV	2×10^6	RNA[s]	linear	2	6.4×10^3

[d] double-stranded nucleic acid
[s] single-stranded nucleic acid

Although the amount of DNA in a procaryotic cell is very small compared with the total amount of DNA in a eucaryotic cell (see table 4.1) this single molecule is very long compared to the size of the cell and it must be very tightly coiled and twisted in order to be accommodated within the cell. The bacterium *Escherichia coli* is only about 2×0.5 μm in size while the total length of the DNA molecule it contains is about 1100 μm, or just over 1mm. We have very little idea as to how a DNA molecule can function when it is coiled up inside a cell several hundreds of times shorter than itself, particularly during replication when the two very tightly coiled strands of the double helix must unwind from around each other.

The Chromosome of *Escherichia coli*

There are two fundamental differences between procaryotic and eucaryotic cells; firstly, procaryotic cells lack certain organelles such as mitochondria and chloroplasts that are typical of eucaryotic cells, and secondly, they do not have a nuclear membrane and so no discrete nucleus. In bacteria the bulk of the cellular DNA, the chromosome, is concentrated in a diffuse body called the *chromatin body* which can be observed in living bacteria by phase contrast microscopy. Electron micrographs have shown that the chromosomal DNA within this chromatin body is attached to an invaginated part of the cell membrane (figure 3.1) and it is believed that this membrane plays an important part in the regular segregation of the two daughter chromosomes whenever the cell divides, achieving the same purpose as the mitotic spindle in higher organisms.

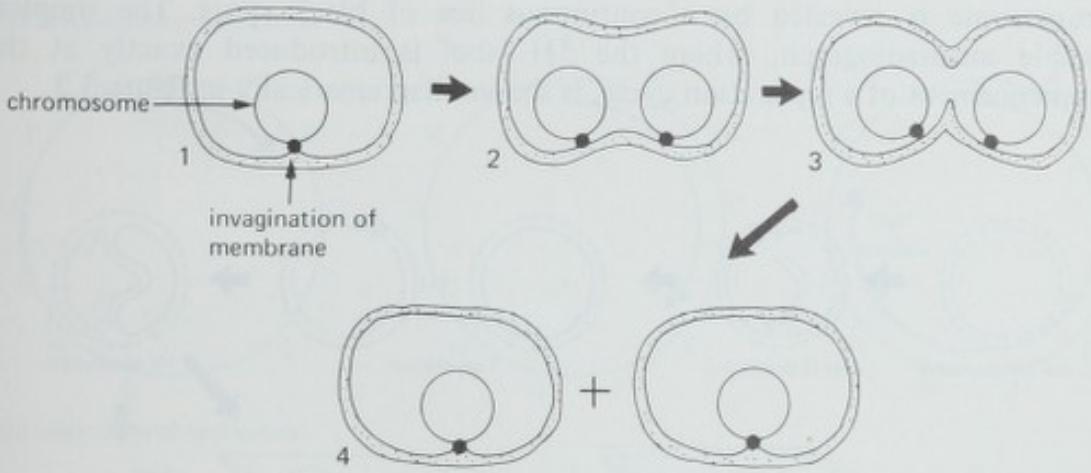


Figure 3.1 The bacterial chromosome is attached to the cell membrane. A non-dividing cell (1) has a single chromosome attached to an invaginated part of the cell membrane. When the chromosome replicates (2) the membrane grows between the separate points of attachment of the daughter chromosomes (3), ensuring that one goes into each daughter cell. Eventually, two daughter bacteria are produced (4) each with a single daughter chromosome attached to its own membrane.

Autoradiography

Initially, the evidence from electron microscopy suggested that all DNA molecules were linear and had two free ends, but as more refined methods were developed for extracting DNA from cells, it became apparent that many DNA molecules, including the chromosome of *E coli,* were circular. The first convincing demonstration that the *E. coli* chromosome was circular was made by John Cairns in 1963, using an autoradiographic technique.

With this technique cells of *E. coli* are grown in a medium containing tritiated thymidine (that is, thymine labelled with radioactive ^{3}H instead of normal ^{2}H)

and allowed to incorporate it into their DNA for about two generations. The cells are then very gently lysed by digesting part of the cell wall with lysozyme and the DNA extracted and allowed to settle very gently onto a dialysis membrane. The membrane is mounted on a microscope slide, overlaid with a photographic emulsion and kept in the dark for up to two months. During this time the ^3H particles undergo radioactive decay and emit beta particles. These beta particles 'expose' the emulsion and after photographic development each emission is recorded as a black spot or grain. Since beta particles have a very low energy and only travel about 1 μm the position of the silver grains in the emulsion is a good indication as to the actual location of the tritium particles in the chromosome. Furthermore, the number of spots on the emulsion is a measure of the density of the tritium label along the underlying molecule of DNA, so that a molecule of DNA labelled with ^3H along both strands will produce twice as many spots as a molecule labelled in only one strand. When the developed slide is examined under a light microscope the size and shape of the chromosome is revealed by a continuous line of black spots. The simplest possible autoradiograph, where the ^3H label is introduced exactly at the commencement of a replication cycle, is shown diagramatically in figure 3.2.

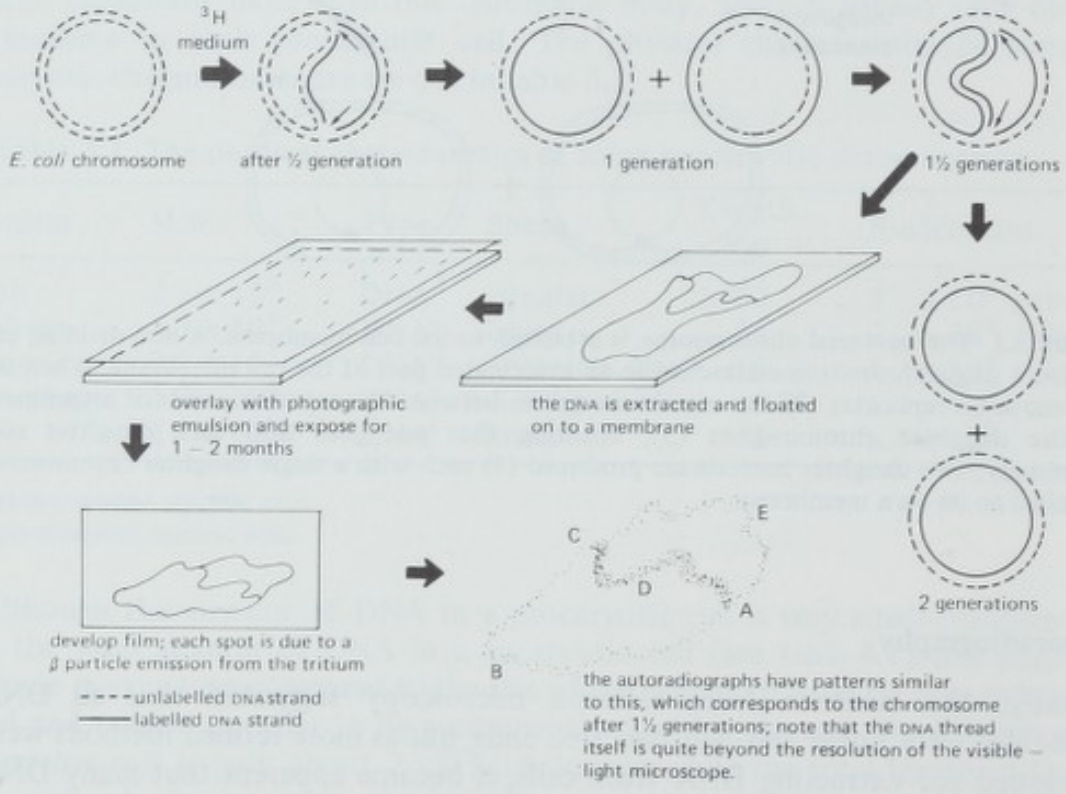


Figure 3.2 Autoradiography of the chromosome of *E. coli*. *E. coli* is grown in a medium containing tritiated (^3H) thymidine and removed after one to two generations; the DNA is extracted, floated onto membranes and autoradiographed. The shape of each replicating molecule is shown by the grain pattern on the X-ray film while the grain density indicates whether one or both strands of the DNA duplex are labelled.

The shapes of the autoradiographs made by Cairns clearly show that the chromosome is circular and that when it replicates it splits into two halves, while the grain densities show that each molecule is two-stranded and so a duplex of DNA. Cairns noted that in each replicating molecule, like the one shown in figure 3.2, the unreplicated section (CBA) was labelled in *one* strand only so that the parental duplex must have had one 'hot' and one 'cold' strand; since one daughter duplex was labelled in only one strand (CEA, 'hot'/'cold') while the other was labelled in both strands (CDA, 'hot'/'hot') replication must have occurred by a semi-conservative mechanism with the strands separating at each point of replication. Cairns correctly deduced that each successive replication cycle commenced from a fixed initiation point but he incorrectly concluded that the chromosome replicates in one direction only. Several experiments have since demonstrated that in *E. coli, Salmonella typhimurium* and *Bacillus subtilis* the chromosome replicates simultaneously in both directions, although they do not

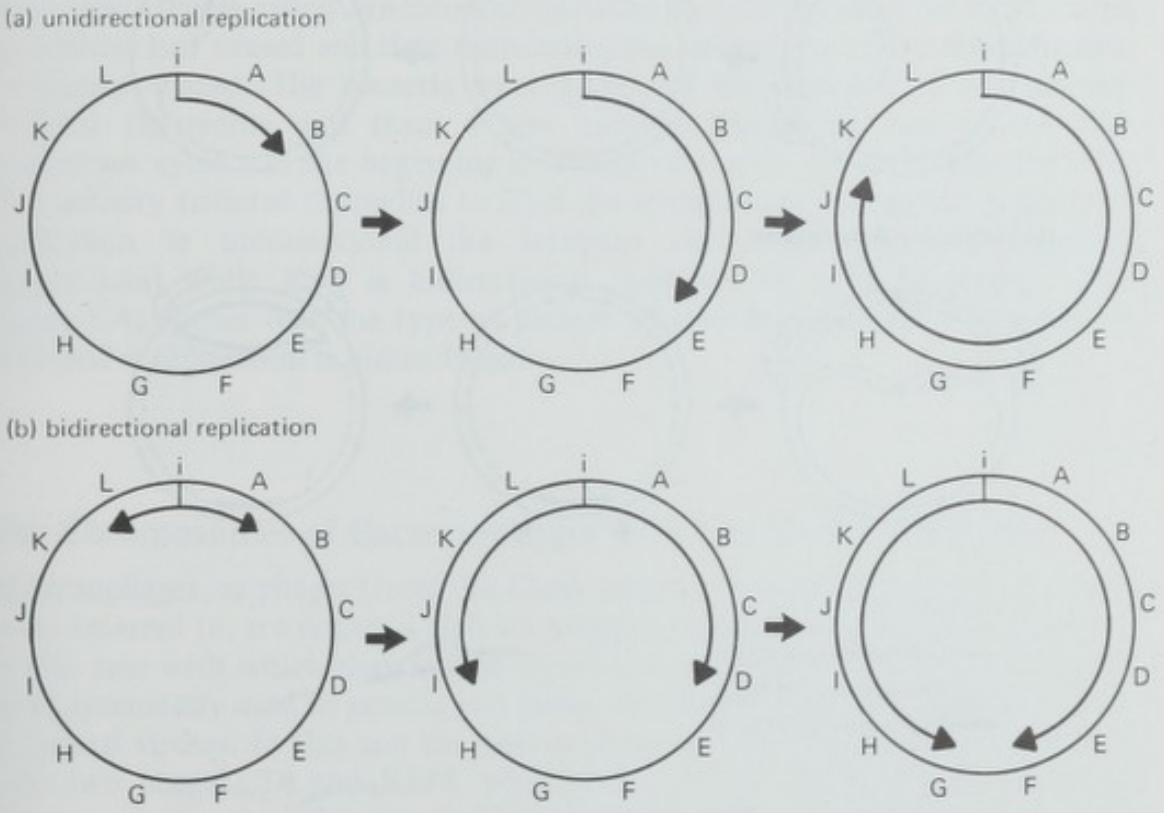


Figure 3.3 Unidirectional and bidirectional replication. In a population of rapidly growing bacteria the replication forks are randomly distributed around the chromosomes; thus while the vast majority of the cells will have replicated genes close to the initiation point, i, only a few will have replicated the genes most remote from i.

If there is unidirectional replication (a) in a clockwise direction A will be the most frequent gene in the population, L the least frequent, with a gradient of decreasing frequency for the intermediate genes.

If there is bidirectional replication (b) there will be two such gradients, from A to F and from L to G.

Note also that with unidirectional replication there is close linkage between the most and the least frequent genes; this is not so with bidirectional replication.

exclude the possibility that *some* chromosomes in a population replicate unidirectionally. The chromosomes of the bacteriophages T4, T7 and λ have also been shown to replicate bidirectionally.

Millicent Masters and Paul Broda (1971) used a genetic method to demonstrate bidirectional replication. The time required to replicate the bacterial chromosome is equal to the generation time of the cell so that in a growing population nearly every chromosome is in the process of replication; since every chromosome commences to replicate at an initiation point (i) most of the cells will have replicated genes close to i but only a few, those at the end of a replication cycle, will have replicated genes remote from i, so that the closer

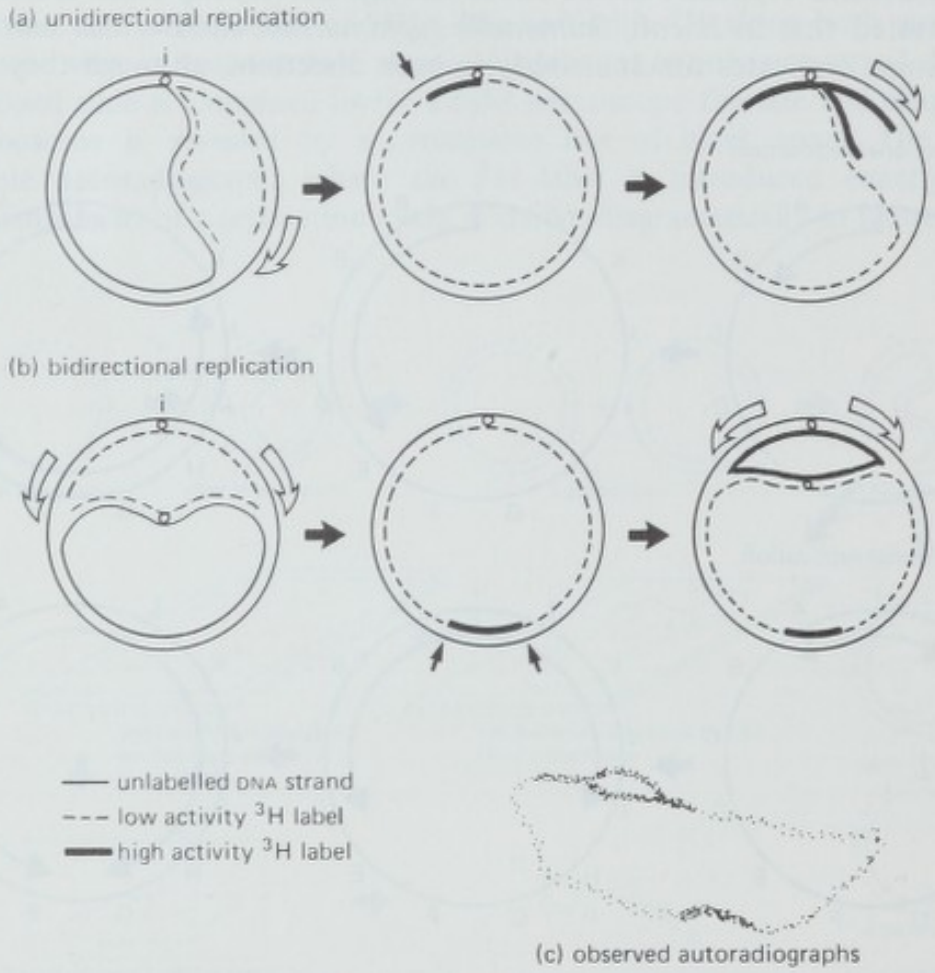


Figure 3.4 Autoradiography of the *E. coli* chromosome. Cells were labelled with low-activity tritiated thymidine at the commencement of a replication cycle, and just before the end of the first replication cycle (small arrows) were transferred to high-activity medium. After a further short period the chromosomes were examined by autoradiography.

If there is unidirectional replication (a) the terminus of the first replication and the initiation point (i) of the second replication will coincide and there will be a single replication fork (open arrows). If there is bidirectional replication there will be two replication forks (b), remote from the terminus of the first replication. The observed autoradiographs (c) are in agreement with bidirectional replication.

Note that only one of the two daughter molecules of the first replication is shown.

a gene to i the more copies of it there will be in the population. If there is unidirectional replication (figure 3.3a) there will be a single gradient of gene frequency around the chromosome and the most and least frequent genes will be closely linked; with bidirectional replication (figure 3.3b) there will be gradients in both directions from i with the most and least frequent genes at opposite points on the chromosome. Masters and Broda estimated the relative frequencies of a series of genes at different positions on the chromosome of *E. coli* by measuring the frequency with which each marker was transferred by the general transducing phage P1 (transduction is described in chapter 7) and obtained results consistent only with bidirectional replication.

Direct autoradiographic visualisation has confirmed that replication is bi-directional. R. L. Rodriguez and his co-workers used a mutant of *E. coli* which when grown at 42° C would complete any existing cycles of replication but would not initiate any new cycles; since this strain grew normally at 25° it was a simple matter to ensure that all cells in the population commenced replication at the same time (this is called synchronous growth) by growing the cells at 42° until replication had ceased and then transferring the cultures to 25° to reinitiate new replication cycles. The bacteria were grown in the presence of *low* activity tritiated thymidine and then, a few minutes before the end of the first replication cycle and the beginning of the second cycle the cells were fed with *high* activity tritiated thymidine to label the terminus and the initiation point. If replication is unidirectional the terminus and origin must be adjacent (figure 3.4a) while if it is bidirectional they will be separate (figure 3.4b). Figure 3.4c shows that the type of pattern of labelling observed is precisely as expected if replication is bidirectional.

The Chromosomes of Bacteriophages with Double-Stranded DNA

Bacteriophages, or phages (from the Greek *phagos,* to consume) as they are more often referred to, are viruses which are obligate parasites on bacteria and because of the ease with which they can be handled in the laboratory have been much more extensively used in genetic and molecular studies than have either the plant or animal viruses. In this and the next section we will principally be concerned with two phages, T4 and X174, which infect *E. coli.* Both these are *virulent* phages, meaning that whenever a phage particle attacks a bacterial cell that cell is inevitably killed and breaks open releasing several hundred mature phage particles.

Phage T4 (T = type) has a complex structure (figure 3.5a, b) and is made up from a protein head enclosing the DNA, a protein tail and six protein tail fibres; many of the larger phages have a similar structure and all have a single molecule of double-stranded DNA for their chromosome. When a particle of T4 infects *E. coli* a complicated sequence of events occurs: (i) the phage attaches by its tail fibres to a special receptor site in the bacterial cell wall, (ii) an enzyme (lysozyme) is released by the tail fibres and bores a hole through the cell wall,

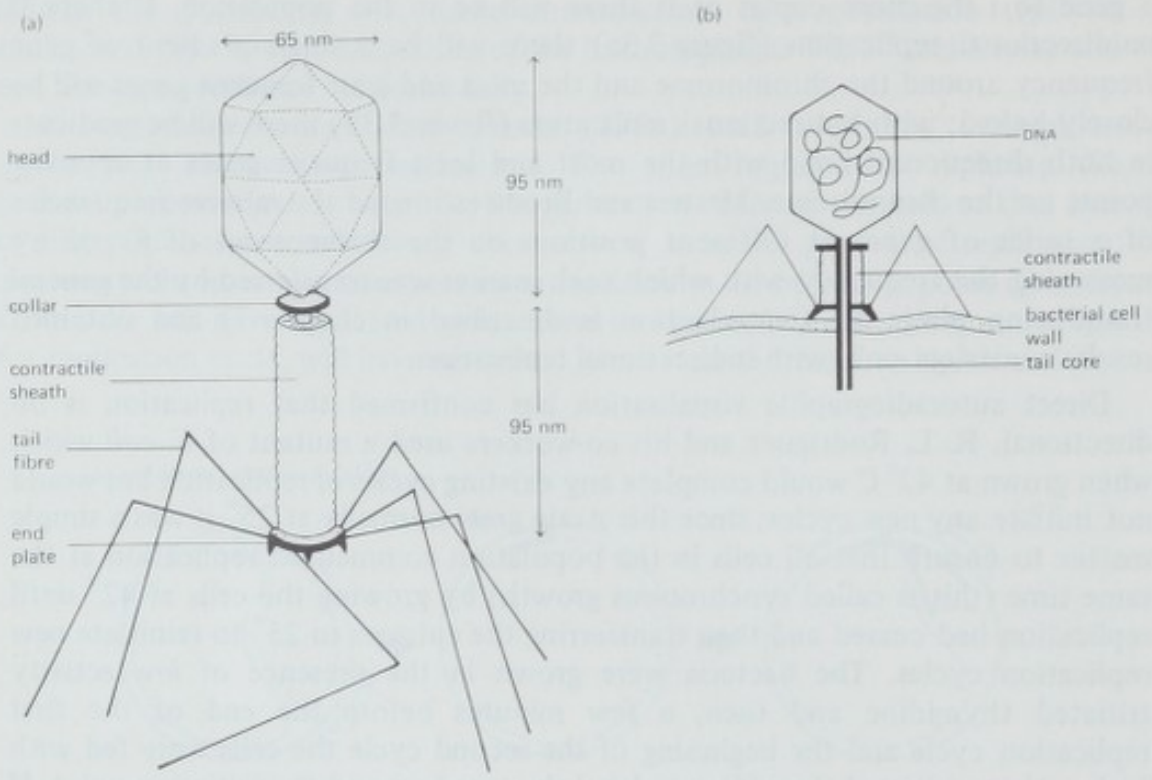


Figure 3.5 Bacteriophage T4.

(a) Phage T4 consists of a protein head, made up of many identical protein subunits and containing a single molecule of DNA, and a complex tail. The protein tail consists of a rigid hollow core, a contractile sheath made from spiral protein and a spiked end plate with a central hole and six attached tail fibres.

(b) Upon infection of *E. coli* the phage attaches by its tail spikes and fibres to a special receptor site on the bacterial cell wall, the tail sheath contracts, the rigid core is 'pushed' through the cell wall and the DNA is injected into the bacterium.

(iii) the contractile sheath contracts, (iv) the rigid tail core penetrates the cell wall, rather like a hypodermic needle, and the phage DNA is injected into the bacterium, (v) the phage DNA takes over the biosynthetic machinery of the host cell, and more phage chromosomes and protein coat components are synthesised, (vi) the various components are assembled into mature and infectious phage particles, and (vii) about 30 minutes after infection the bacterial cell breaks open (lyses) and the phage particles are released. This is the *lytic cycle* which is obligatory for a virulent phage.

If a virus particle is gently disrupted all the DNA (or RNA) can be isolated as a single molecule and this, because of its small size, can be directly visualised by electron microscopy or by the Cairns autoradiographic technique. While these methods reveal a great deal about the morphology of the viral chromosome, such as the shape of the molecule (whether it is linear or circular) and its overall length, they may not give us any information about particular nucleotide sequences within the molecule. This is important as it turns out that these chromosomes sometimes show variations in structure that are not usually

observed in DNA molecules from other sources; they may be terminally redundant, circularly permuted, or they may have cohesive ends.

A terminally redundant molecule has the sequence of base pairs at one end of the molecule repeated at the other end; each of the four molecules represented in figure 3.6d is terminally redundant. The simplest way of understanding the meaning of circular permutation is to imagine that each linear molecule of DNA is produced by cutting a circle of DNA at random points (figure 3.6e); thus while each molecule contains a complete set of nucleotide pairs it starts at a random point determined by the position of the cut. An unlimited number of cyclically permuted molecules are possible by breaking open a circle of DNA in this way. A molecule of DNA is said to have cohesive ends (figure 3.10) when its opposite ends are single-stranded and base complementary. Just how the T4 chromosome replicates inside the *E. coli* host cell is still very poorly understood, but replication proceeds together with recombination (chapter 16) and the immediate product is not a number of separate linear molecules but a long end-to-end continuum, or *concatenate*, of identical DNA 'molecules' each corresponding to one complete set of phage genes (a set of phage genes is represented by the ten letters a to j in figure 3.6). When this DNA is packaged

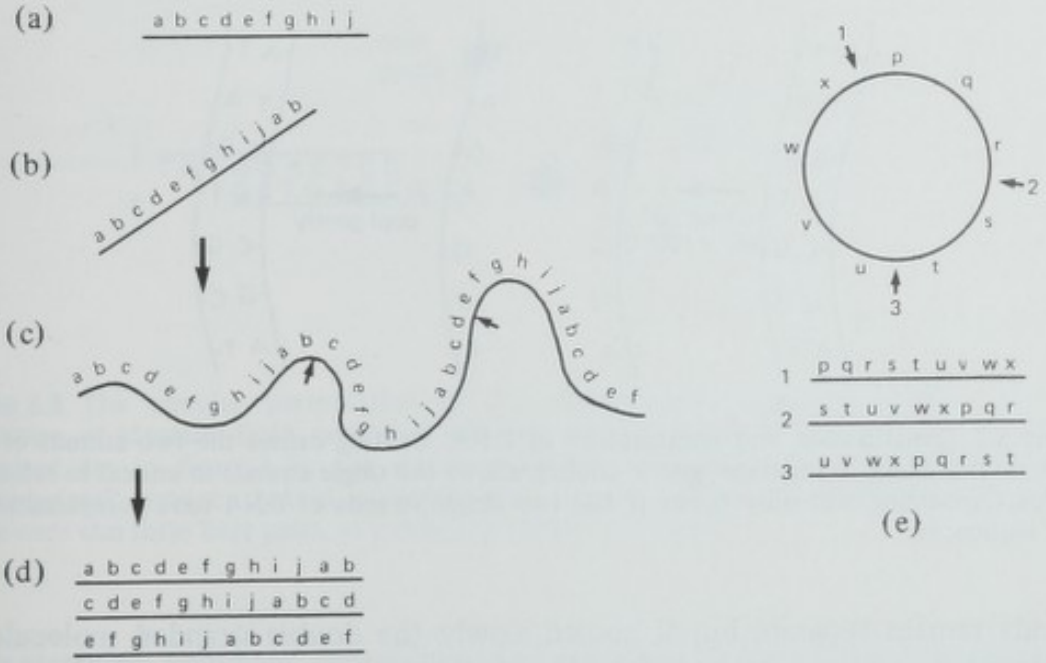


Figure 3.6 Terminal redundancy and circular permutation.

(a) A unit set of genetic information, represented by the ten letters a to j.

(b) The infecting phage chromosome is greater than unit length (twelve letters), and the nucleotide sequence at one end of the molecule is repeated at the other end.

(c) Replication and recombination produce a long length of DNA with repeating nucleotide sequences.

(d) Each phage head accommodates 'twelve letters' of DNA. The DNA is cut into pieces of this length, indicated by the arrows in (c). The phage chromosomes produced are both terminally redundant and circularly permuted.

(e) Circular permutation without terminal redundancy is the result of cutting open a circular molecule of DNA at different points.

into phage heads to produce mature phage particles an unknown mechanism cuts off a constant amount of DNA from the concatenate; this amount of DNA exactly fills one phage head and is just more than one complete set of genes (figure 3.6c and d). The result is that the sequence of nucleotide pairs at one end of each molecule is duplicated at the other end; each molecule of phage DNA, that is each phage chromosome, is terminally redundant. Furthermore, because each piece of DNA severed from the concatenate (twelve letters long in figure 3.6) is greater than unit size, each successive molecule will start and end with a different nucleotide sequence–the molecules are also circularly permuted.

The amount of terminal redundancy varies between 0.5 and 3 per cent of the genome, or between 200 and 1150 nucleotide pairs.

DNA/DNA Hybridisation

These features of phage chromosomes were first demonstrated by using a DNA denaturation and renaturation technique. When a solution of DNA is heated to 80–100° C the hydrogen bonds between the bases break and the two strands of the duplex separate (figure 3.7); if the heated solution is cooled rapidly the

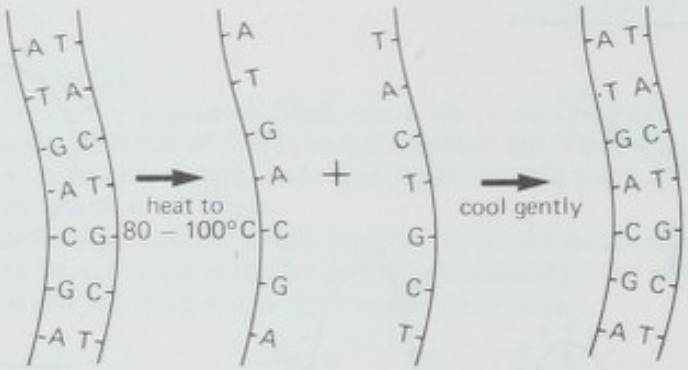


Figure 3.7 Denaturation and renaturation of DNA. Heating causes the two strands of the DNA double helix to separate–gentle cooling allows the single strands to anneal to reform a duplex. Annealing will only occur if the two single strands of DNA have complementary base sequences.

strands remain separate but if cooled slowly the double-stranded molecule is renatured by the reforming of hydrogen bonds between the complementary strands of DNA. This technique of melting and annealing DNA molecules has proved an invaluable tool in studying genetics at the molecular level, as two pieces of single-stranded DNA will only anneal if they have stretches of complementary or nearly complementary base sequences; the degree of hybrid DNA molecule formation when two species of denatured DNA are cooled together is a measure of their similarity. This technique has also been extensively used in examining the relatedness of DNA and RNA by making DNA/RNA hybrids, where one strand of a renatured double helix is DNA and the other strand is RNA.

Circular Permutation and Terminal Redundancy

To demonstrate that the T2 chromosome is circularly permuted, Charles A. Thomas denatured a population of chromosomes and allowed them to reanneal. If only one molecular species of DNA were present, annealing would reform the original duplices and only rod-shaped molecules would be produced; but if the chromosomes were circularly permuted then the complementary regions of two circularly permuted strands can anneal (figure 3.8) producing a molecule with

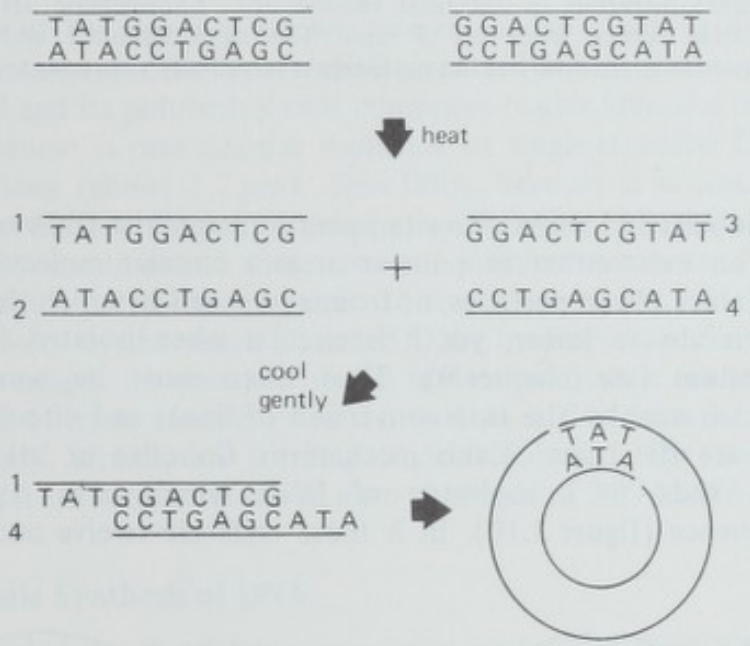


Figure 3.8 The circular permutation of T2 chromosomes. The denaturation and renaturation of identical DNA molecules will only produce linear molecules similar to those denatured (as in figure 3.7); the denaturation of a population of circularly permuted molecules will produce hybrid molecules with single-stranded base complementary ends. These ends can form base pairs, so producing circular molecules.

single-stranded and base complementary end; these ends could then unite to form a circular molecule. When Thomas examined the renatured DNA under the electron microscope he observed these circular molecules. The demonstration of terminal redundancy was even simpler; molecules of T2 DNA were treated with exonuclease III which attacks both strands of the DNA duplex from the 3′ ends (figure 3.9) leaving short single-stranded extremities. If the molecules are terminally redundant, but not otherwise, these single-stranded extremities will be base complementary and the molecules will circularise. Once again Thomas was able to detect circular molecules. Thus the chromosome of T2 is both terminally redundant and circularly permuted.

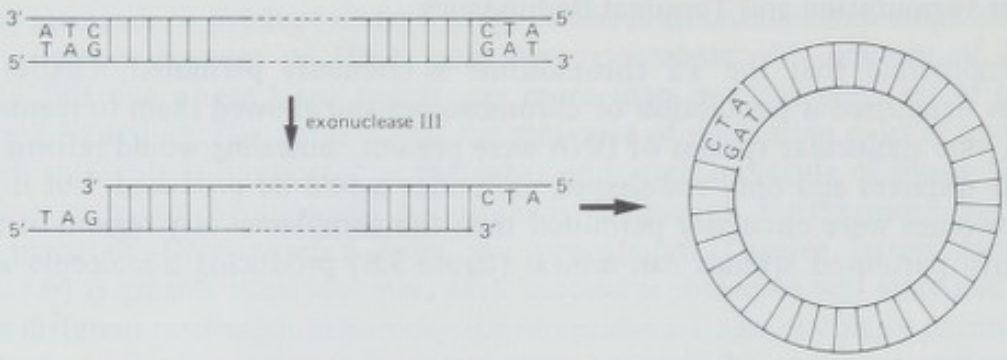


Figure 3.9 The demonstration of terminal redundancy. Exonuclease III degrades each strand of the DNA duplex from the 3′ end. The single-stranded ends will be base complementary and circularise *only* if the molecule is terminally redundant.

Cohesive Ends

In phage λ (lambda) and some other temperate phages the DNA comprising the chromosome can exist either as a linear or as a circular molecule. The DNA isolated from intact phage particles, or from a host cell in which the phage DNA is replicating, is always linear, yet it is circular when isolated from a newly infected bacterium (see chapter 9). Thus there must be some molecular mechanism which enables the interconversion of linear and circular molecules; cohesive ends are the basis of this mechanism. Cohesive or 'sticky' ends are single-stranded ends to a molecule of DNA which have complementary nucleotide sequence (figure 3.10). In λ these ends are twelve nucleotides long

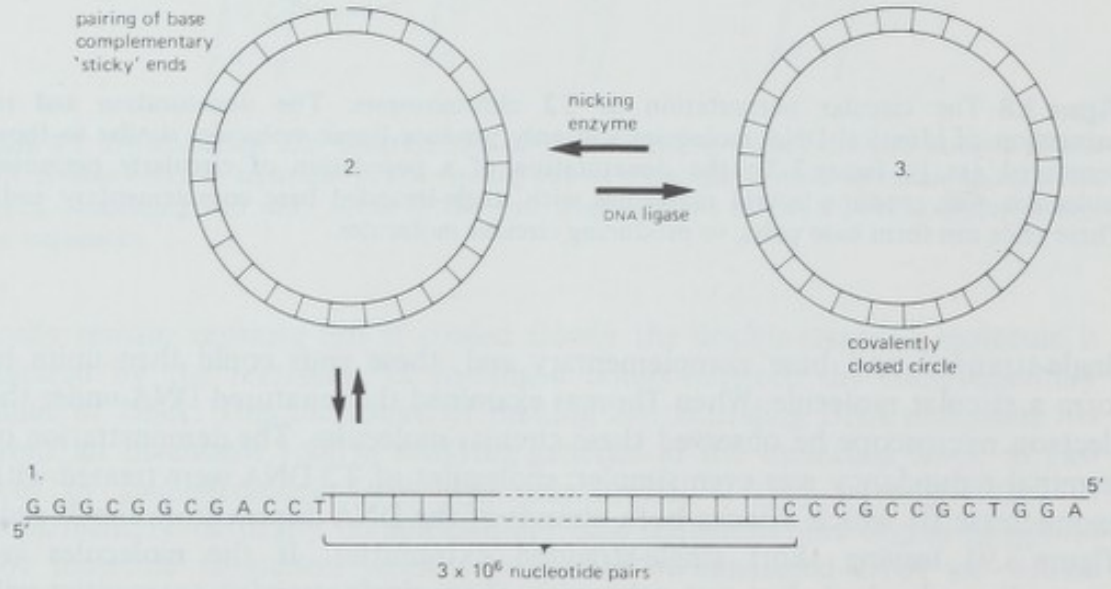


Figure 3.10 The cohesive ends of phage λ. The extremities of the linear chromosome have single-stranded base complementary ends (1)—these sticky ends can anneal to form a circular DNA duplex (2) and the gap can be sealed by DNA ligase to form a covalently bonded circle of DNA. The diagram shows the actual sequence of nucleotides in the sticky ends.

and the exact sequence of nucleotides in them is known. Thus at any time the molecule can circularise simply by the pairing of the complementary bases, and the gaps can be sealed by an enzyme (DNA ligase) to form an intact or covalently bonded circle. Linear molecules are formed from circles by the reverse process, similar except that both strands of a covalently bonded molecule must be 'nicked' by a specific enzyme before the strands can separate.

The Chromosome of X174—A Phage with Single-Stranded DNA

X174 is one of the smallest known viruses (about 20nm in diameter) and its structure is more typical of some small plant and animal viruses than of a phage. It has no tail and its polyhedral coat comprises twelve identical tubular subunits. The chromosome is one circular molecule of single-stranded DNA only 5000 nucleotides long (about 1.7 μm). This DNA, because it is single-stranded, has several properties not characteristic of normal double helical DNA; for example, since the bases are unpaired there are not equivalent amounts of A and T and of C and G, and it is particularly sensitive to reaction with formaldehyde.

The discovery of this single-stranded DNA by Robert L. Sinsheimer in 1959 immediately raised the question how it replicates—does it act as a template for the synthesis of more identical strands, or does it first serve as a template for a complementary strand, so forming a double helix? The answer was soon forthcoming from studies on the *in vitro* synthesis of X174 DNA.

The Enzymatic Synthesis of DNA

The *in vitro* synthesis of DNA was pioneered by Arthur Kornberg and his co-workers in 1956. They found that they could synthesise limited amounts of DNA in a synthetic system containing: (i) the four deoxynucleoside *tri*phosphates, dATP, dGTP, dCTP and dTTP; (ii) the enzyme DNA polymerase, purified from cell-free extracts of *E. coli*—this enzyme was the first to be discovered that is involved in DNA synthesis and it extends nucleotide chains in the 5′ to 3′ direction by forming the 3′-5′ phosphodiester bonds; (iii) Mg^{2+} ions; and (iv) a high molecular weight DNA template.

Several lines of evidence strongly suggested that this synthesis was not just a random polymerisation of the nucleotides present in the reaction mixture but rather was a replication process, accurately copying the template DNA. First, no DNA could be synthesised if the template DNA was omitted; second, provided that all the four nucleotide triphosphates were present their relative concentrations had no effect on the composition of the product DNA; third, although it was not possible to determine the nucleotide sequences of high molecular weight DNA the available evidence indicated that the template and the product DNA were probably identical—for example, the relative frequencies of A-T and C-G base pairs were always identical in the template and in the product, irrespective of the source of the template DNA (table 3.2).

Table 3.2 The base compositions (per cent) of template and enzymatically synthesised DNA

DNA Source	Template		Product	
	A + T	C + G	A + T	C + G
E. coli	49	51	51	49
calf thymus	56	44	60	40
Mycobacterium	32	68	36	64
T2	64	36	64	36

In these experiments the template DNA was double-stranded—what happens if instead we use single-stranded X174 DNA as the template? When there was only a limited (20 per cent) increase in the amount of DNA (that is, there were no second generation synthetic molecules present) the product DNA contained a T for every A in the primer, a C for every G, and so on; clearly, the first DNA to be synthesised was single-stranded and base complementary to the single-stranded template DNA (table 3.3). With extensive (600 per cent) synthesis the base ratios corresponded to those expected with the replication of double-stranded DNA, and there were equivalent amounts of A and T and of C and G. *In vitro* it appears that the single strand of X174 DNA (referred to as the (+) or viral strand) first becomes double-stranded by the synthesis of the complementary (–) strand; this double-stranded molecule then acts as a template for further double-stranded molecules.

Table 3.3 The base compositions (per cent) of X174 template DNA and the product DNA

	A	T	C	G
X174 template DNA	25	33	24	18
Product DNA:				
20 per cent synthesis	31	24	20	25
600 per cent synthesis	27	29	23	21

Although great care is necessary in extrapolating from behaviour *in vitro* to behaviour *in vivo*, all the evidence indicates that *in vivo* the X174 chromosome replicates in the same way. When the viral (+) strand enters the host cell it first becomes attached to the cell membrane and if this attachment fails the chromosome is unable to replicate (figure 3.11). The complementary (–) strand is synthesised, forming a double-stranded circular molecule, the replicative form (RF), and this repeatedly replicates by a semi-conservative mechanism. At one time it was thought that only the molecule containing the membrane-bound viral (+) strand could continue to replicate and that the other daughter molecule did not replicate further but simply acted as a template for the production of messenger RNA, but it now appears that both daughter molecules replicate and

continue to replicate until 20–50 double-stranded RF molecules have been synthesised. When this semi-conservative replication ceases the double-stranded RF molecules repeatedly serve as templates for new single-stranded (+) DNA. Finally, the viral (+) strands become encased in protein coats forming mature X174 particles.

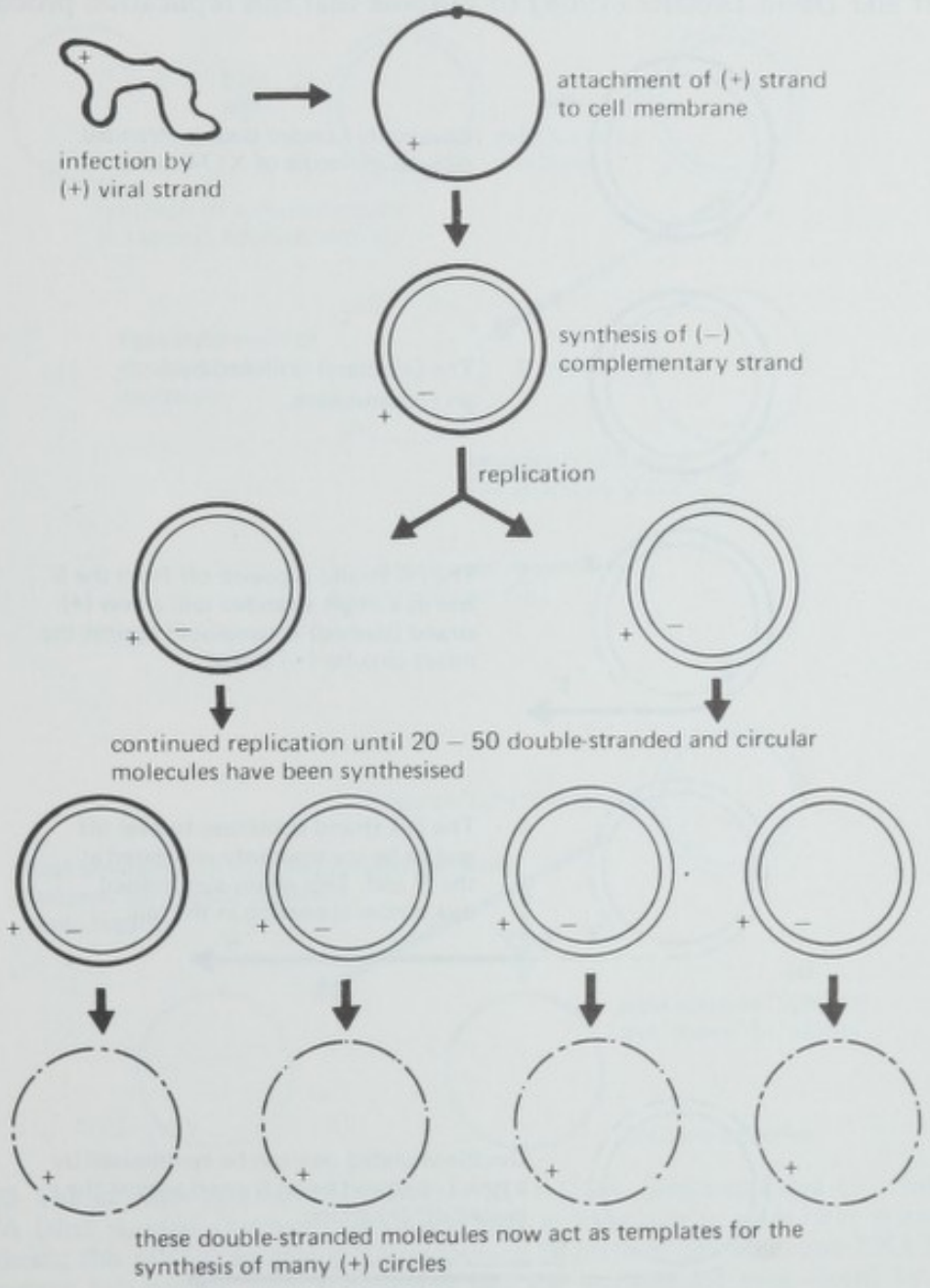


Figure 3.11 The *in vivo* synthesis of ϕX174 DNA. The infecting (+) circle of single-stranded DNA attaches to the membrane of the infected bacterium and a complementary (–) strand is synthesised. This double-stranded molecule replicates repeatedly until twenty to fifty molecules of double-stranded DNA have been produced; these daughter molecules now act as templates for the production of single-stranded (+) circles, the viral chromosomes.

The Rolling-Circle Model

We do not yet fully understand how the double-stranded RF molecule act as a template for the single-stranded daughter molecule, but when these single-stranded products are first synthesised they are found as concatenates which are not only considerably longer than the unit length of a chromosome but are also linearly continuous with the parental DNA. These observations prompted Walter Gilbert and David Dressler (1968) to propose that this replication proceeded by

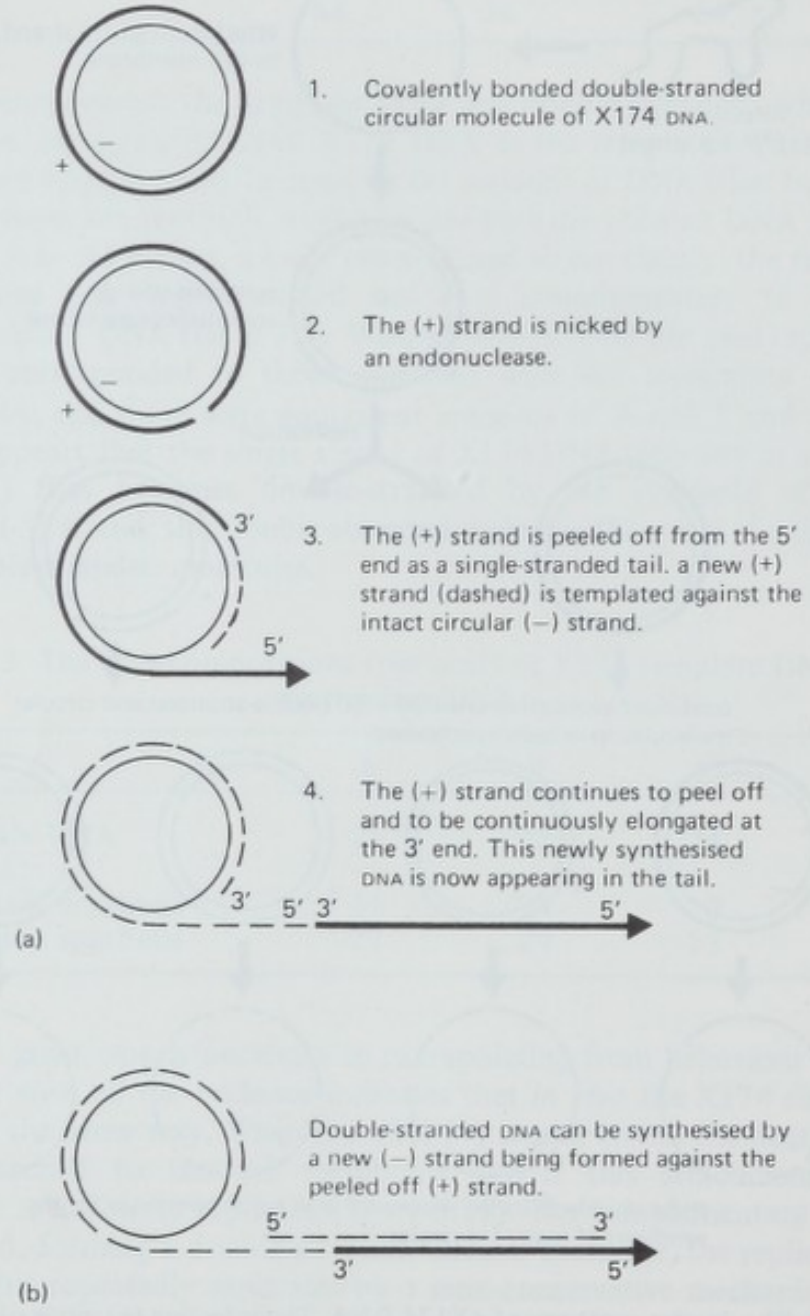


Figure 3.12 The rolling-circle model for X174 replication.
(a) One way that single-stranded (+) molecules can be produced from a double-stranded circular template.
(b) The production of double-stranded DNA by the rolling-circle mechanism.

a *rolling-circle* mechanism. Figure 3.12 shows one of the possible ways by which a rolling-circle model can account for this asymmetric replication. A single-stranded break occurs in the (+) strand of the covalently bonded circular molecule (RF I), presumably as a result of endonuclease action, and the original (+) strand is peeled off at the 5′ end as a single-stranded tail. As the (+) strand is peeled off so a new (+) strand is formed by complementary base pairing with the

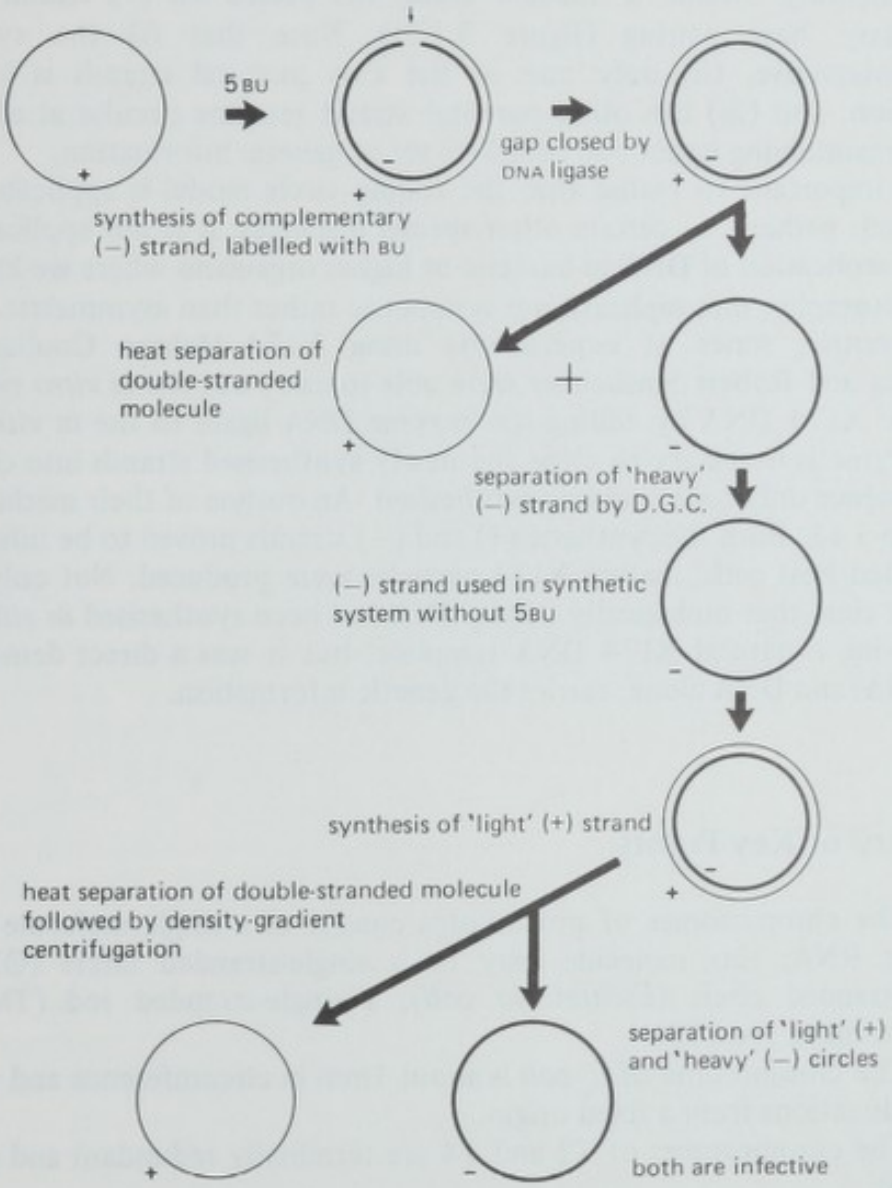


Figure 3.13 The *in vitro* synthesis of infective X174 DNA. Single-stranded (+) circles of X174 DNA (that is, viral chromosomes) were used as templates in an *in vitro* system for DNA synthesis; this system contained 5-bromouracil in place of thymine, and DNA ligase. Complementary (–) strands were synthesised and the single-strand gaps closed by DNA ligase to form covalently bonded circles of DNA. These double-stranded circles were denatured and the heavy (–) strands separated by density-gradient centrifugation; these (–) strands were then used as templates in a system containing thymine (and no bromouracil). Complementary (+) strands were synthesised and the light (+) and heavy (–) strands isolated by denaturation followed by density gradient centrifugation. Both the (+) and (–) strands were infective and produced mature X174 particles in an *E. coli* host.

intact circular (–) strand (figure 3.12a). Thus the new (+) strands are formed by a process which continuously elongates the parental (+) strand.

There is now an increasing body of evidence not only supporting the rolling circle model but suggesting that the initial replication of the double-stranded DNA may also proceed by a rolling-circle mechanism. A double-stranded molecule is presumed to replicate in much the same way except that a new (–) complementary strand is formed along the peeled off (+) strand by complementary base pairing (figure 3.12b). Note that (i) the synthesis is semi-conservative, (ii) only one of the two parental strands is involved in replication, and (iii) the other parental strand remains circular at all times, so always maintaining intact one complete set of genetic information.

It is important to realise that the rolling circle model is applicable only to X174 and, perhaps, in certain other special instances; it is not applicable to the normal replication of DNA in bacteria or higher organisms where we know, from autoradiography, that replication is symmetric rather than asymmetric.

In another series of experiments using X174 Mehran Goulian, Arthur Kornberg and Robert Sinsheimer were able to carry out the *in vitro* synthesis of *infective* X174 DNA by adding the enzyme DNA ligase to the *in vitro* system; this enzyme is necessary to close the newly synthesised strands into circles, and in its absence only rods could be synthesised. An outline of their method is given in figure 3.13. Both the synthetic (+) and (–) strands proved to be infective and, in infected host cells, mature X174 particles were produced. Not only was this the first time that biologically active DNA had been synthesised *in vitro*, even if by copying a natural X174 DNA template, but it was a direct demonstration that DNA, and DNA alone, carries the genetic information.

Summary of Key Points

(1) The chromosomes of procaryotes consist of a single molecule of either DNA or RNA; this molecule may be a single-stranded circle (ØX174), a double-stranded circle (*Escherichia coli*), a single-stranded rod (TMV) or a double-stranded rod (T2).

(2) The chromosome of *E. coli* is about 1mm in circumference and replicates in both directions from a fixed origin.

(3) The chromosomes of T2 and T4 are terminally redundant and circularly permuted.

(4) The linear chromosome of phage λ has single-stranded and base-complementary ends. These cohesive ends enable the molecule to circularise immediately after infection.

(5) ØX174 chromosomes are circles of single-stranded DNA. After infection this single-stranded molecule attaches to the bacterial membrane and becomes double-stranded by the synthesis of a complementary strand. These double-stranded circles replicate more double-stranded circles which then act as templates for the synthesis of many single-stranded circles of infective DNA.

(6) Infectious X174 DNA has been synthesised *in vitro* by copying natural X174 DNA.

(7) Special techniques described are: (i) autoradiography of the bacterial chromosome; (ii) annealing of base complementary single-stranded DNA; (iii) enzymatic synthesis of DNA.

4 The Chromosomes of Eucaryotes

Read not to contradict and confute, nor to believe and take for granted, nor to find talk and discourse, but to weigh and consider.
Francis Bacon (1561-1626)

When any cell divides, the continuity of the genetic material must be maintained and each daughter cell must receive an exact copy of each and every chromosome. Not only must the chromosome duplicate exactly but there must be a precise mechanism which ensures that one replica of each chromosome is transmitted to each daughter cell—if this mechanism fails there will be too many or too few chromosomes in the daughter cells.

In procaryotes the chromosome is simply a molecule of DNA which replicates semi-conservatively from a single initiation point. In eucaryotes the chromosome is a highly complex organelle and in addition to specific proteins may contain over 400 times the amount of DNA in the chromosome of *E. coli.* Although we have reason to believe that a eucaryotic chromosome is one very long length of DNA folded back and forth upon itself and held together by specific protein molecules, we still have little idea as to just how these molecules are arranged within the compact structure of a chromosome. This lack of knowledge makes it difficult to explain chromosome replication in molecular terms; nevertheless it is clear that the continuity of the DNA could be maintained by a mechanism of semi-conservative replication, even though there are obvious mechanical difficulties in replicating such a long length of DNA and ensuring the regular segregation of the two daughter chromosomes.

Although we do not know how replication is achieved at the molecular level, we do know that there is a special process of cell division during which these very long chromosomes condense and become visible under the light microscope. The gross morphology of the chromosomes during cell division is most conveniently studied by staining an actively dividing tissue, such as a root tip, with a chromosome specific dye such as leuco-basic fuchsin; this reacts specifically with aldehyde groups released from DNA after mild acid hydrolysis and not only stains the chromosomes but can also be used to measure quantitatively the amount of DNA they contain. For most of the cell cycle (figure 4.1) the chromosomes are involved in heterocatalytic activity and produce the ribonucleic acids required for protein synthesis; this is *interphase* (the $G_1 + S + G_2$ periods) and it is during this time (the S period) that the DNA replicates. The interphase nucleus presents a granular appearance and the chromosomes are not visible as such but are spun out into long threads and seen only as a diffuse mass of *chromatin;* within each nucleus are one or more clearer areas, the *nucleoli.* It is only as cell division proceeds that the chromosomes condense and, by progressive coiling, become the highly compacted structures seen during cell division; how the chromosomes condense is not known.

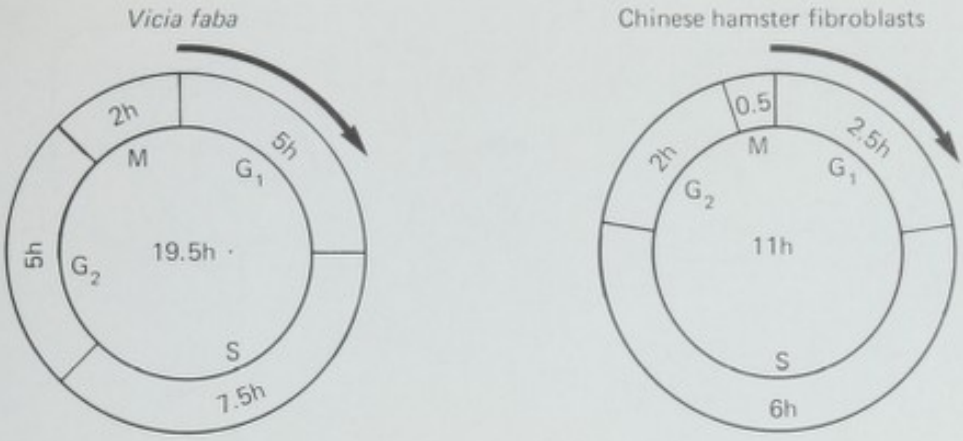


Figure 4.1 Cell cycles. The cell cycle is divided into four phases which may be of different durations in different organisms: G_1: Pre-DNA synthesis (1st *G*ap); S: period of DNA synthesis; G_2: post-DNA synthesis (2nd *G*ap); M: mitosis.

After the DNA has replicated and the chromosomes have condensed to the maximum extent, each chromosome is seen to be split along its length into two identical sister *chromatids,* except at a special region called the *centromere* where the two sister chromatids remain held together. The centromere remains relatively uncondensed and so appears under the light microscope as a constriction in the continuity of the pair of sister chromatids. The centromere, also known as the primary constriction, is a highly specialised region of the chromosome and has two special functions; first, it holds together the two sister chromatids until it is time for them to separate and, second, it is the point on the chromosome to which the spindle fibres will eventually attach. In addition, each haploid set of chromosomes has at least one secondary constriction, the *nucleolar organiser*; this region, which controls the formation of the nucleolus (the function of the nucleolar organiser region is discussed in chapter 18), appears as a gap in the chromatids but under the electron microscope it is seen to be traversed by a thin uncoiled strand of chromatin. Finally, we can note that some chromosomes, or parts of chromosomes, remain compacted or condensed during all or most of the cell cycle; these regions are termed *heterochromatic* ('differently coloured') regions to distinguish them from the *euchromatic* ('true coloured') regions which show the typical coiling and uncoiling pattern referred to above. It is thought that the heterochromatic regions are functionally inert.

Cell Division

For the convenience of description the process of cell division or *mitosis* (from the Greek *mitos,* a thread) is divided into a number of stages, but it must be remembered that mitosis is a continuous living process and that there is no fixed point at which a cell passes from one stage to the next. Mitosis in a generalised animal cell is illustrated in figure 4.2.

At the onset of mitosis, *prophase,* the nucleolus breaks down and the chromosomes become individually recognisable; they can then be seen to be divided longitudinally into two sister chromatids. As prophase proceeds the

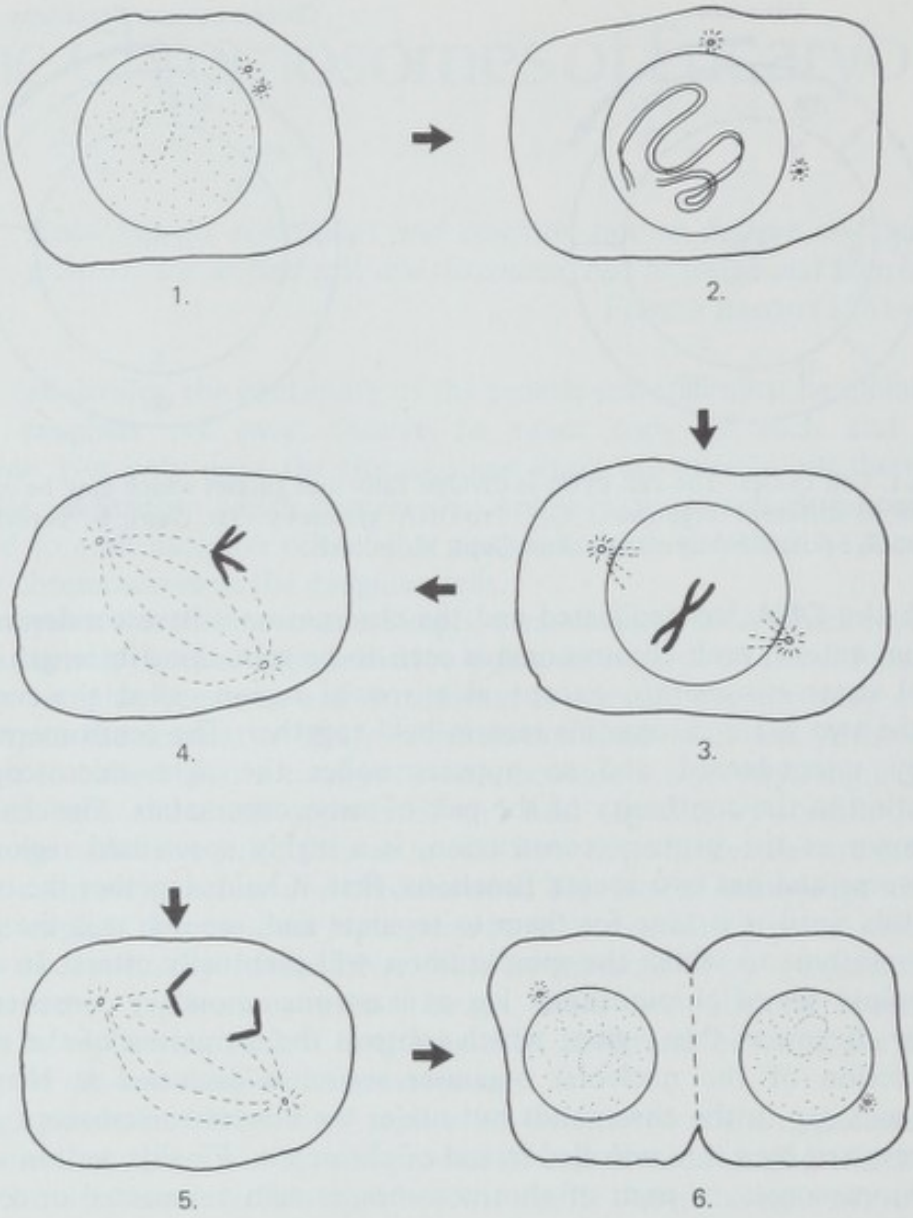


Figure 4.2 Mitosis in a generalised animal cell. For the sake of clarity only one chromosome is shown. Every other chromosome will behave in exactly the same way.

(1) Resting stage. The chromosomes are not visible and the nucleus has a granular appearance, with a clearer region, the nucleolus. The centriole is already double.

(2) Prophase. The chromosomes are seen as discrete entities, divided longitudinally into two sister chromatids. The nucleolus has broken down.

(3) Later prophase. The chromosomes continue to condense, and centrioles have moved to the poles.

(4) Metaphase. The mitotic spindle has formed from the centrioles. The nuclear membrane has broken down, and the centromeres attach to the mid-points of the spindle fibres.

(5) Anaphase. Each centromere divides and the spindle fibres contract, so drawing the daughter chromosomes towards the poles.

(6) Telophase. The spindle breaks down, the nucleolus and the nuclear membrane reform and the chromosomes despiralise, forming two resting-stage nuclei. A new cell membrane forms between the daughter cells.

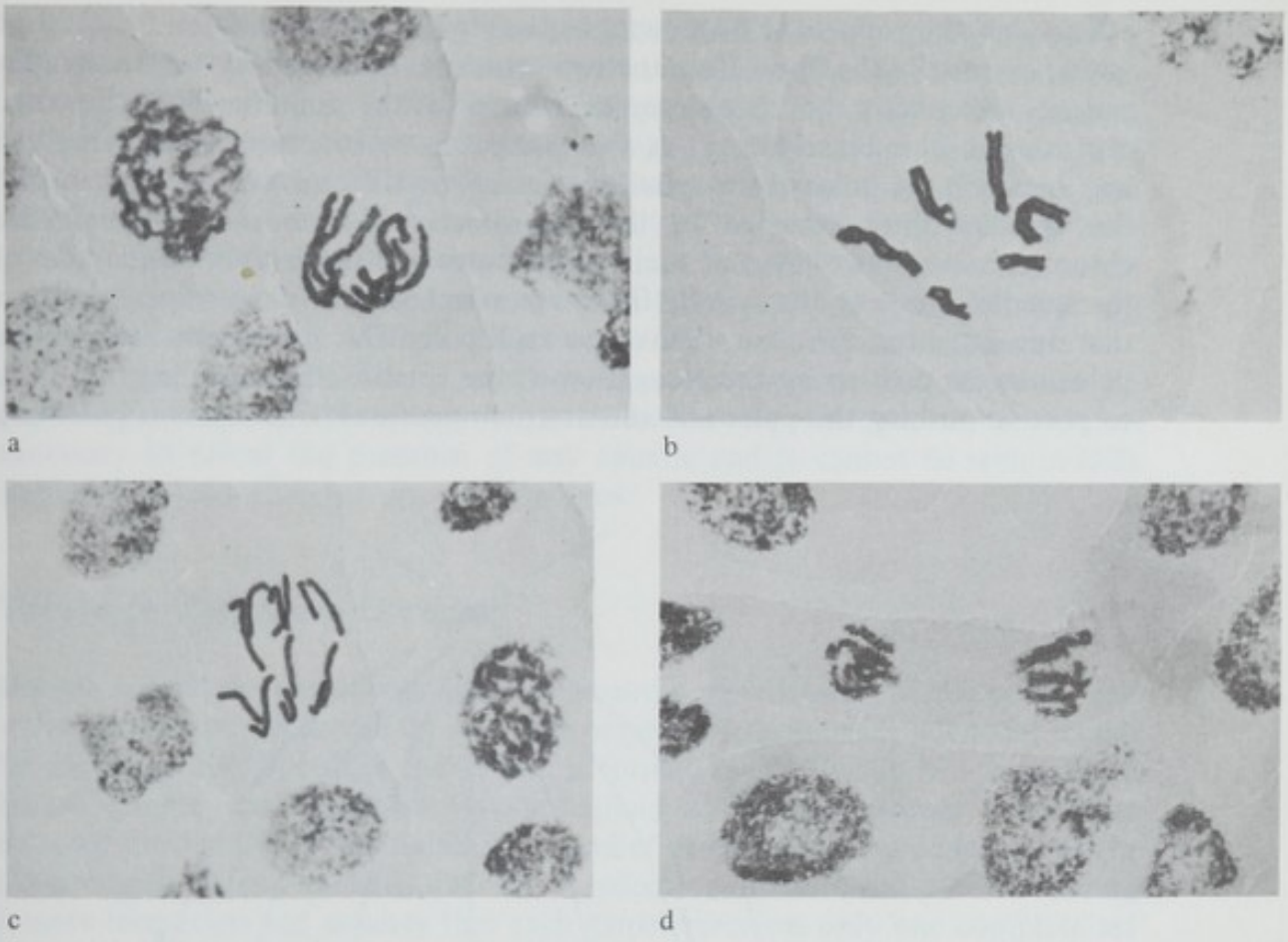


Figure 4.3 Mitosis in *Crepis capillaris* (Hawk's Beard)
(a) *Prophase* The nucleus at the left is in early prophase and the chromosomes have started to condense but are not yet individually recognisable. The nucleus at the right shows a later stage with the chromosomes more fully contracted and clearly seen to be divided along their length into two chromatids.
(b) *Metaphase* The mitotic spindle has formed (this cannot be seen) with the poles at 11 o'clock and 5 o'clock and the six chromosomes are orientated on the metaphase plate. The chromosomes are attached to the spindle fibres by their centromeres, seen as small constrictions near the inner ends of the chromosomes.
(c) *Anaphase* The centromeres have divided and a set of six daughter chromosomes is moving towards each of the poles of the spindle.
(d) *Telophase* The sets of daughter chromosomes have congregated at the poles of the spindle and have started to despiralise. It is at this stage that the nuclear membrane re-forms, a new cell wall is laid down and the nuclei re-enter the resting stage. The lower nuclei, with a granular appearance, are in resting stage.

[The author is most grateful to Dr Neil Jones for making these preparations and photographs.]

chromosomes continue to condense with the sister chromatids still held together by the centromeres. Meanwhile the two centrioles have moved to opposite sides of the nucleus where they form the poles of the mitotic *spindle*, a very important structure that ensures the regular segregation of the daughter chromosomes. At the end of prophase the nuclear membrane breaks down and the spindle forms by a number of very fine microtubules being rearranged in the

cell so that they appear to be growing outwards from the centrioles, forming the asters; eventually the fibres from the two centrioles coalesce to form the spindle, and at *metaphase* the chromosomes attach to the spindle fibres by their centromeres in a plane known as the *metaphase* or *equatorial plate* lying half way between the poles of the spindle. At *anaphase* the centromeres separate and the spindle fibres attached to the centromeres shorten so that the sister chromatids are drawn apart at their centromeres and move to opposite poles of the spindle. The way the spindle fibres are attached to the centromeres ensures that one daughter centromere moves to each pole. This movement towards the poles may be assisted by the elongation of the spindle fibres running from pole to pole so pushing the poles and the sister chromatids farther apart (figure 4.3).

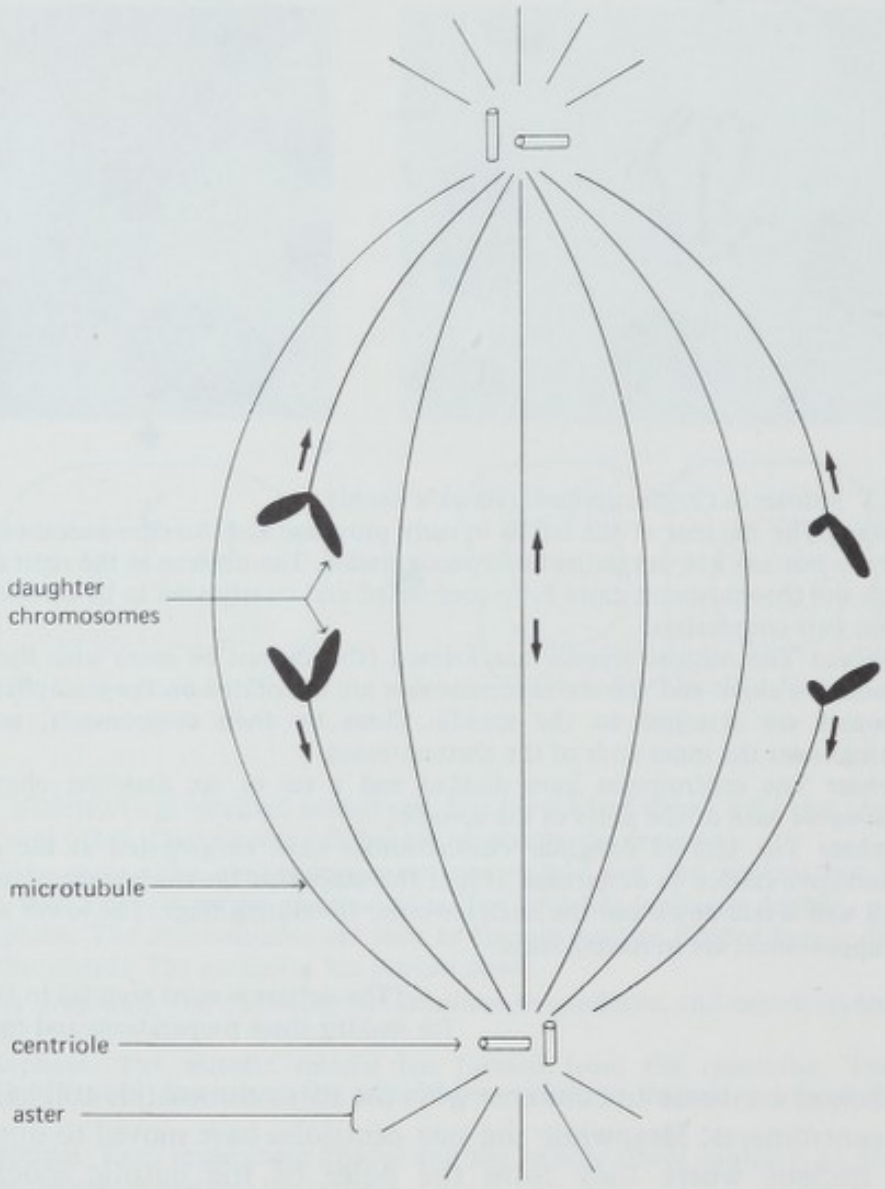


Figure 4.4 The mitotic spindle. The chromosomes move to the poles (i) by a shortening of the fibres attached to the centromeres, and (ii) by an elongation of the fibres running continuously from pole to pole.

Note that once the centromeres have separated each sister chromatid becomes a separate sister chromosome. The result is that one complete set of chromosomes is assembled at each pole of the spindle. The process of cell division is completed by the breakdown of the spindle, the re-formation of the nuclear membrane and of the nucleoli, the uncoiling of the chromosomes and the formation of a new cell membrane between the two daughter cells. This is *telophase.* We now have two daughter cells, each with exactly the same chromosome complement as the mother cell. This is the normal process of cell division by which the fertilised egg (*zygote*) divides so that it can develop into an adult multicellular organism.

In higher plants mitosis is similar (figure 4.4) except that there are no centrioles and the spindle simply forms between two poles. Special methods are necessary to reveal the presence of any spindle and it cannot be seen in cells stained only with chromosome-specific dyes.

Meiosis or Reduction Division

Mitosis accurately reproduces the chromosome complement of the parent cell and ensures that every cell in an adult organism is genetically identical to the fertilised egg cell, but since the zygote is formed by the fusion of a male and a female gamete, each contributing one haploid set of chromosomes, there must be some mechanism which halves the number of chromosomes at some stage in the life cycle. This process is *meiosis,* or reduction division, and it occurs during gamete formation and ensures that each gamete receives only one complete set of chromosomes; thus when the two gametes fuse to form the zygote there are again two complete sets of chromosomes (that is, the zygote is diploid). In the absence of meiosis the number of chromosomes would double at each successive generation.

The essence of meiosis is that it consists of *two* divisions of the cell but only *one* division of the chromosomes, so halving the number of chromosomes. But meiosis has another very important function as it permits the *recombination* of the genetic material. The following account describes meiosis in a generalised plant or animal cell, but it must be remembered that the details of the process may show considerable variation from one species to another.

The First Meiotic Division

When the chromosomes first become visible at prophase they are long and filamentous and, although we know that the DNA has already duplicated, they are not yet divided into chromatids. These chromosomes have a rather bumpy appearance, rather like beads on a string, and it is believed that each bump or *chromomere* corresponds to one gene. The homologous chromosomes now come together and pair, or synapse, zipperwise along their length (figure 4.5(1) and (2)) and at the same time they condense and so appear thicker; each pair of chromosomes is now referred to as *a bivalent.* Each chromosome now divides into two chromatids (figure 4.5(3)) and although the homologous chromosomes

are apparently repelling each other they are held together by a *chiasma* or by several chiasmata (figure 4.5(4)). Each chiasma has arisen as if by the breakage at exactly corresponding points of two *non-sister* chromatids and their reunion in a new combination, so that genetic material has been physically exchanged between these two chromatids; this is *recombination* or *crossing-over* (figure 4.5(5); see also figure 5.7). The exchange of genetic material and the formation of chiasmata is an essential part of meiosis; if in a particular bivalent no chiasma forms there is nothing to hold the homologous chromosomes together as a bivalent, and the chromosomes cannot align correctly on the metaphase plate.

The chromosomes now condense further by coiling and the chiasmata terminalise—that is to say, they are pushed to the ends of the bivalent, in the way that a twist between two pieces of string moves towards one end when the other ends are pulled apart. This is the end of *prophase.*

The nuclear membrane now breaks down, a spindle forms and each bivalent attaches to the spindle so that homologous centromeres lie on opposite sides of the metaphase plate (figure 4.5(6)); this is *metaphase I.* At *anaphase I* (figure 4.5(7)) the spindle fibres contract, as during mitosis, and one centromere from each bivalent is drawn to each pole. Observe that the centromeres do *not* separate as they do at mitosis. At *telophase I* (figure 4.5(8)) the nuclear membranes reform and the cell divides into two. This is the end of the first meiotic division, and there may now be a resting stage before the commencement of the second meiotic division, which takes place in each of the two products of the first meiotic division.

The Second Meiotic Division

At the onset of the second meiotic division, the chromosomes are already divided into chromatids and they immediately align on the metaphase plates of newly formed spindles (figure 4.6(1)); this is *metaphase II.* At *anaphase II* (figure 4.6(2)) the centromeres separate and one daughter chromosome moves to each pole. Finally, at *telophase II,* the interphase (resting stage) nuclei are reformed, resulting in the production of four haploid cells (figure 4.6(3)). Each

Figure 4.5 The first meiotic division. This and the next figure illustrate the behaviour of a pair of homologous chromosomes at meiosis. The inner circle represents the nuclear membrane (when present) and the small circles, the centromeres.

(1) Prophase. The chromosomes become visible and pair along their length.

(2) Pairing is complete.

(3) The chromosomes divide into chromatids.

(4) A chiasma forms by the breakage of non-sister chromatids and their rejoining in a new combination. The two chromosomes are together referred to as a bivalent.

(5) The chromosomes contract further and repel each other; the bivalent is maintained only because of the chiasma.

(6) Metaphase I. A spindle forms (as in mitosis) and each bivalent is orientated on the metaphase plate. The chiasma terminalises and is pushed to the end of the bivalent.

(7) Anaphase I. The homologous centromeres are drawn to opposite poles of the spindle.

(8) Telophase I. The resting-stage nuclei are re-formed and a membrane forms between the two daughter nuclei, forming a diad.

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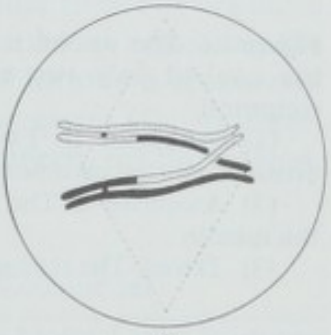

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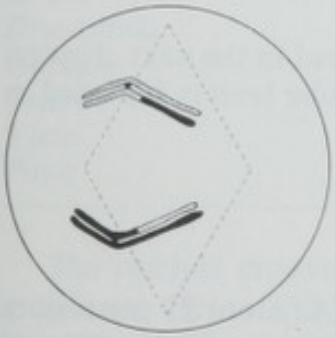

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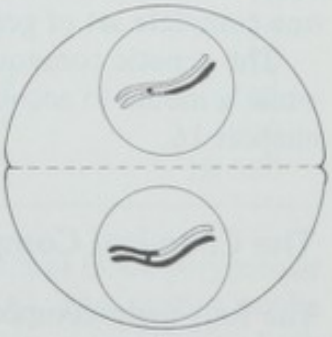

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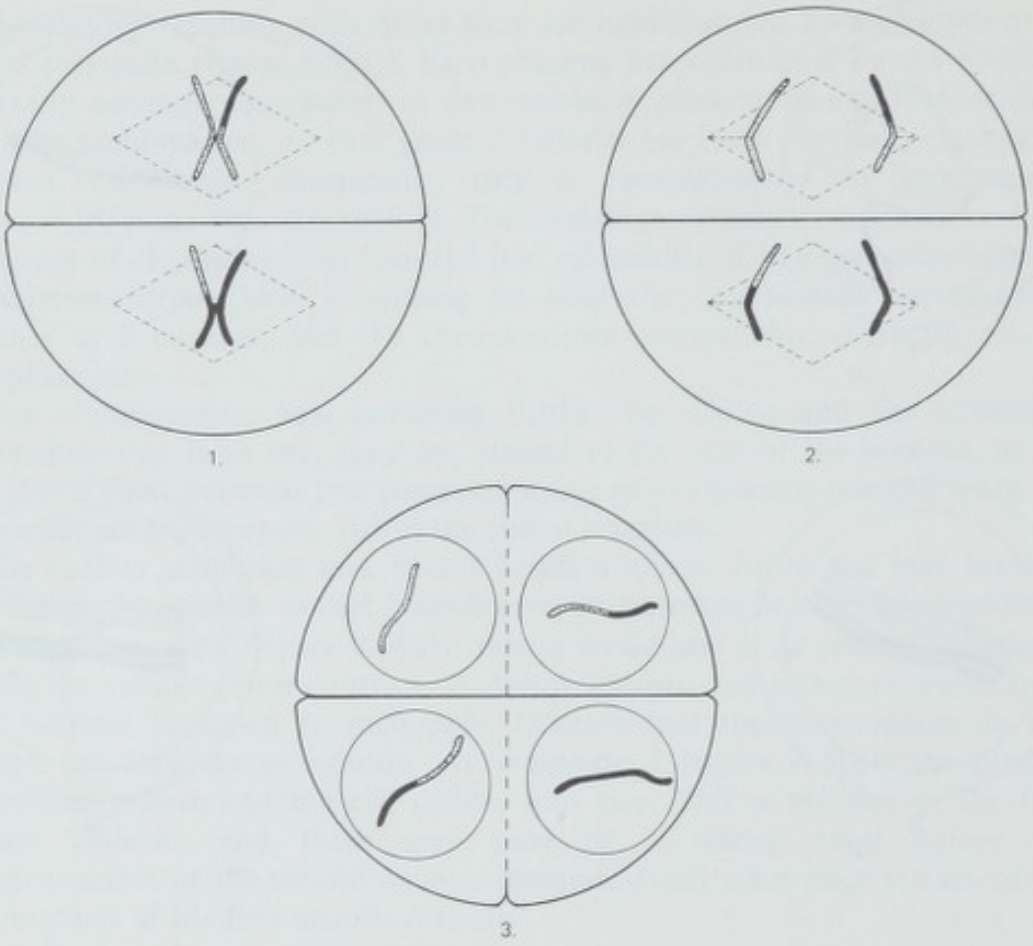


Figure 4.6 The second meiotic division. Note that each recombination event (cross-over) has involved only two of the four meiotic products and that the exchange is exactly reciprocal.

(1) Metaphase II. Two spindles form in the opposite plane to that in the first meiotic division, and the chromosomes orient on them.

(2) Anaphase II. The centromeres separate and one chromosome moves to each pole of the spindle.

(3) Tetrad. The resting-stage nuclei are reconstituted and a new cell membrane forms.

group of four haploid cells resulting from meiosis in one parent cell is referred to as a *tetrad*. Note that in each tetrad, for each recombination event there are two chromosomes which have not been involved in recombination and two which have, and that because the process is exactly reciprocal each meiotic product has one complete set of genetic information.

The genetic consequences of recombination are described in the next chapter, while a model to account for recombination at the molecular level is discussed in chapter 16.

The Chemical Composition of Chromosomes

The four main components of the chromosomes are DNA (about 27 per cent in an 'average' chromosome), basic and residual protein (66 per cent) and RNA (6

per cent). The DNA is the genetic material (chapter 2) and, although every cell in any organism contains the same amount of DNA, the amount of DNA per haploid chromosome set varies enormously from one species to another and does not bear any obvious relationship to the complexity of the organism; thus a pine tree genome may have ten times as much DNA as a human genome (table 4.1). (The total amount of DNA in a human body weighs about 100 g; Ernest DuPraw estimates this DNA to be $1.5\text{-}3.0 \times 10^{13}$ metres long, enough to reach to the sun and back 100 times.) The reasons for this paradox are not yet understood.

The basic proteins, usually histones, are comparatively small molecules (M.W. 10 000–20 000) which can attach to DNA by attractions between the positive charges on their basic groups and the negative charges on the phosphate groups of the DNA. We know that the histones are associated with the wide groove along the DNA double helix (the wide and narrow grooves in DNA are clearly shown in figure 2.8) but it is not known whether they are simply coiled around the DNA double helix or whether they cross-link, and so bind together, adjacent duplices of DNA. This nucleo–histone complex comprises 60–90 per cent of the bulk of the chromosomes. The precise function of the histones is not understood, but if the histones are removed from an isolated chromosome its morphological integrity is maintained, although there is a twofold increase in length, suggesting that the histones are involved in the packaging of the DNA. Further, in an *in vitro* system for RNA synthesis (chapter 12) using chromosomal material (chromatin) as a template the rate of RNA synthesis is increased if the histone is first removed from the chromatin—thus histone may play a role in regulating the activity of DNA. However, there are only a very limited number of histones, and they are remarkably similar from one organism to another, so that it is unlikely that they play direct roles in regulating the activity of *particular* genes (chapter 18). The amount of histone is reasonably constant from one cell to another, although in certain sperm (for example, salmon sperm) they are replaced by a group of related proteins, the protamines.

Table 4.1 The DNA content per haploid chromosome set

Organism	Weight (g)	Nucleotide pairs	Length (mm)
Phage T2	2×10^{-16}	3×10^{5}	0.05
E. coli	4×10^{-15}	3×10^{6}	1.1
Drosophila	9×10^{-14}	8×10^{7}	24
Humans	3×10^{-12}	3×10^{9}	870
Frog	2.4×10^{-11}	2.3×10^{10}	6700
Vicia	2×10^{-11}	2×10^{10}	6000
Pinus	3×10^{-11}	3×10^{10}	10 500

The residual proteins include a number of enzymes such as DNA polymerase (replicates DNA) and RNA polymerase (transcribes DNA), and various acidic proteins. Unlike the histones the residual proteins may vary considerably, both

qualitatively and quantitatively, according to the cell type and its metabolic activity.

The RNA is not a structural component of the chromosomes and although it is synthesised on the chromosomes most of it is transmitted to the cytoplasm where it will play one or other of the roles in protein synthesis. The chromosomal RNA and the residual protein may complex to form another nucleoprotein so that the simplest chemical interpretation of the chromosome is that it is a complex of the two kinds of nucleoprotein.

The Structure of the Eucaryotic Chromosome

Exactly how the nucleoproteins are organised into a chromosome is still one of the mysteries of molecular genetics. Whereas the procaryotic chromosome is a single molecular of nucleic acid visible only by electron microscopy, the eucaryotic chromosome is large enough to be visible under the light microscope, and at times even with a hand lens. Just how many molecules of DNA are contained in a eucaryotic chromosome is an open question.

Electron micrographs of both interphase nuclei and metaphase chromosomes reveal long bumpy fibres, typically about 40 nm in diameter. At one time it was thought that each fibre was made up of two or four smaller fibrils, but more recent and better evidence suggests that these fibres are the basic units of chromosome structure and that each consists of a single DNA double helix tightly coiled inside a protein coat. For example, Ernest DuPraw found that when individual fibres from honey-bee chromosomes were treated with a weak solution of trypsin the protein coats were digested away leaving single trypsin-resistant strands with the dimensions of a DNA double helix. The examination of whole metaphase chromosomes by DuPraw did not reveal any division of the chromatids into half or quarter chromatids, and the absence of free fibre ends suggested that each chromatid consisted of one long fibre.

The strongest evidence that the chromosome contains one long fibre, and so just one long length of DNA, comes from autoradiographic experiments first carried out by J. Herbert Taylor in 1957. The reasoning behind this experiment is similar to that used by Meselson and Stahl (chapter 2), the most important difference being that whereas the latter investigated the replication of *molecules* of DNA, Taylor studied the replication of *whole chromosomes.* He grew seedlings of the broad bean, *Vicia faba,* in tritiated thymidine so that any DNA formed during replication was labelled. After eight hours, when the DNA was fully labelled, the roots were transferred to a normal medium containing colchicine; this prevents the formation of the spindle so that the chromosomes divide but not the cell. Thus, not only are the sister chromosomes retained in the same cell but the number of chromosomes present indicates the number of replication cycles that have occurred. The cells were stained and mounted on microscope slides in the usual way except that they were covered with a photographic emulsion sensitive to the beta particles emitted by the isotope. The preparations were stored in the dark, each beta emission exposing a spot on the

photographic emulsion. After development of the emulsion, the pattern of spots, indicating the distribution of the isotope, could be compared with the actual underlying chromosomes.

After one generation cycle in ^{3}H thymidine, both chromatids of each metaphase chromosome were found to be labelled (figure 4.7). After one further

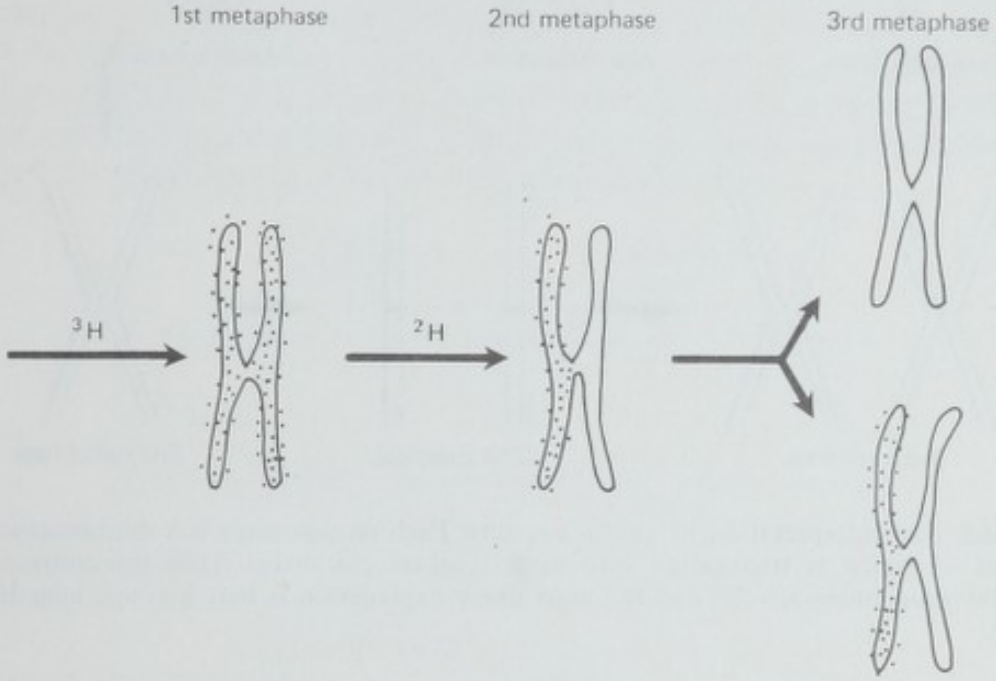


Figure 4.7 Taylor's experiment with *Vicia faba*. Chromosomes were replicated for one generation in tritiated thymidine and then transferred to normal medium. Staining showed the position of each chromatid while the spots indicated which chromatids had a radioactive label.

generation in normal medium each metaphase chromosome had one labelled chromatid; after another generation half the chromosomes were unlabelled and half had one labelled and one unlabelled chromatid. The simplest explanation is that the prereplication chromosome is a duplex structure, one part of which passes into each of the two sister chromatids; thus at the first metaphase each chromatid will consist of one labelled and one unlabelled strand (figure 4.8). After replication in normal medium, each metaphase chromosome will have one chromatid labelled in one strand only and one chromatid without label, and so on. Quite clearly the chromosome is replicating semi-conservatively and is behaving exactly as would be expected if it were one very long length of DNA.

Figure 4.9, based on a model suggested by DuPraw, illustrates one way that the DNA could be organised in a metaphase chromosome.

If we are correct in our interpretations, then the fundamental structure of a eucaryotic chromosome is, like a procaryotic chromosome, one very long length of DNA duplex. However, it is abundantly clear that a eucaryotic chromosome could not replicate like the *E. coli* chromosome from a single fixed initiation point. The *E. coli* chromosome is 1 mm long and takes forty minutes to

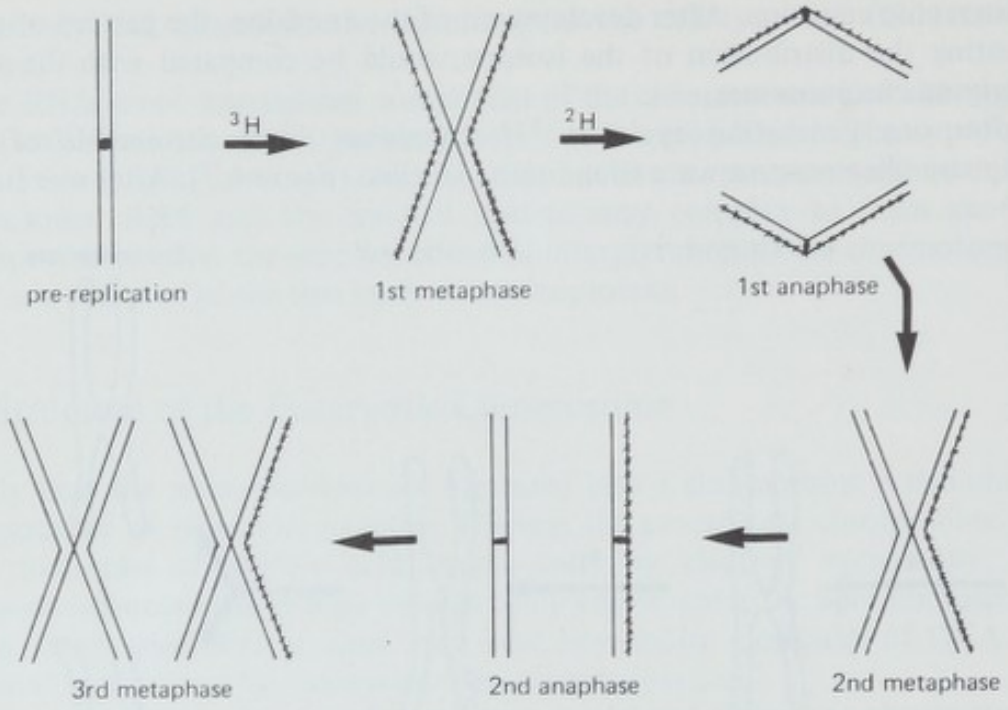


Figure 4.8 The interpretation of Taylor's results. Each chromosome is a double structure, one part of which is transmitted into each daughter chromatid. Thus the chromosome replicates semi-conservatively, and the most likely explanation is that it is one long duplex of DNA.

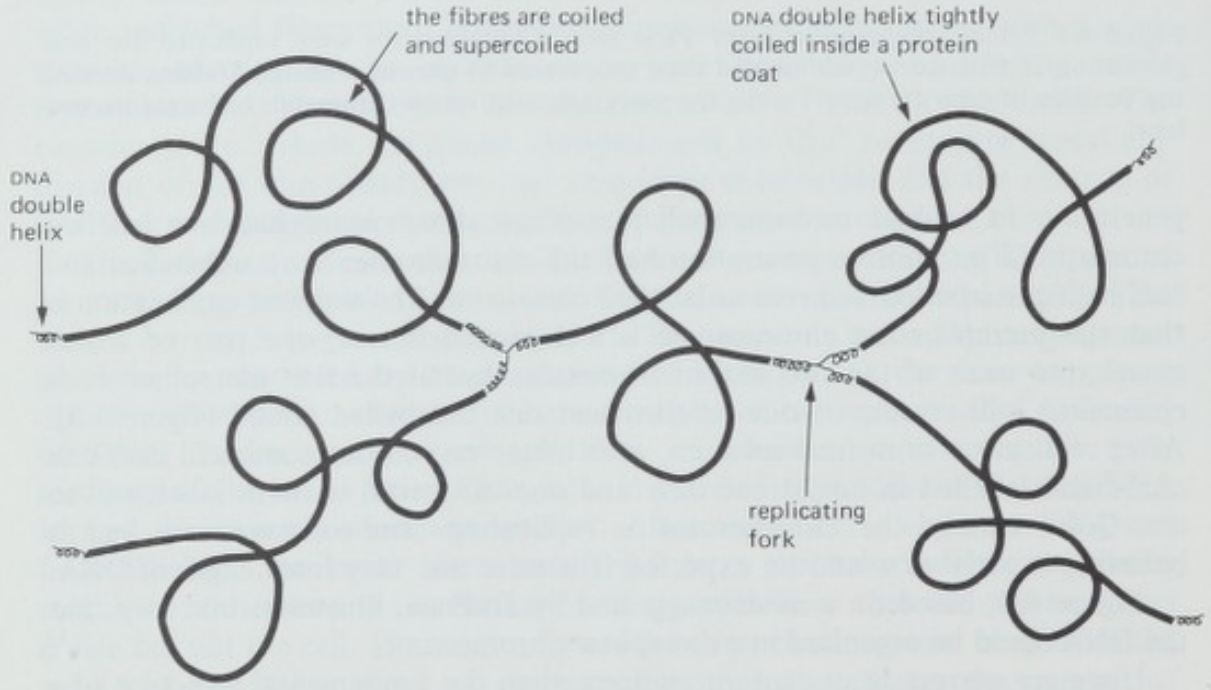


Figure 4.9 The possible structure of a eucaryotic chromosome. According to this model the chromosome is a tightly coiled double helix of DNA in a protein coat; this is the 30–40 nm fibre. The chromosome in the diagram is replicating from two replication forks by a semi-conservative mechanism for DNA replication.

replicate; with bidirectional replication this is a rate of about 15 μm per minute. The average chromosome of *Vicia faba* has about 1 metre of DNA, and if it replicated at 15 μm per minute in both directions and from a fixed initiation point the process would take about twenty-four days instead of the observed five hours (the length of the S period). The replication of DNA from the chromosomes of the Chinese Hamster has been studied using autoradiography by Joel Huberman and Arthur Riggs, and their results show that each molecule of DNA does indeed replicate from a number of points and in both directions. Each replicating segment was between 7 and 30 μm long and the rate of replication was about 0.8 μm per minute, so that a replicating segment would take between ten and forty minutes to complete replication (figure 4.10).

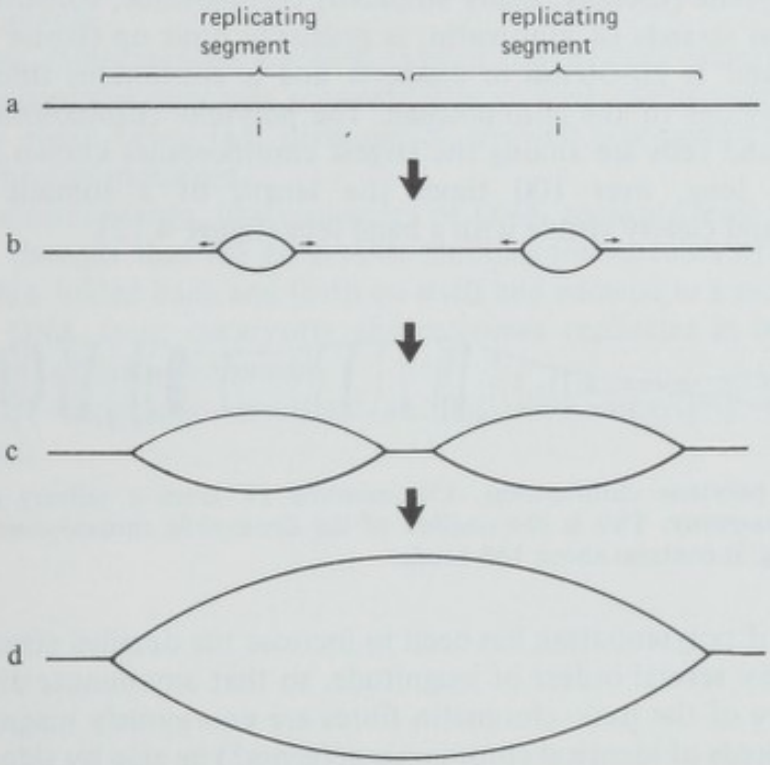


Figure 4.10 The replication of eucaryotic DNA. In the Chinese hamster the DNA replicates in both directions from a number of fixed initiation points (i):
(a) the brackets show two adjacent replicating segments, each about 20 μm long;
(b) replication has commenced from both initiation points and proceeds in both directions at about 0.8 μm per minute;
(c) replication is nearing completion;
(d) when both adjacent segments have completed replicating two continuous lengths of daughter DNA are produced.

Polytene Chromosomes

In certain tissues of Dipteran larvae the process of cell division is arrested although the cells continue to grow by increasing in size. In these tissues, notably the salivary glands, the homologous chromosomes pair along their length and coil around each other; this somatic pairing is similar to the synapsis that

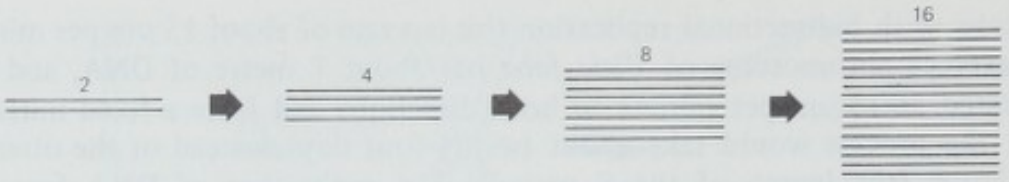


Figure 4.11 Polytenisation. Two homologous chromosomes pair and replicate repeatedly without separation of the daughter chromatids so building up a giant multistranded chromosome with 4, 8, 16, and so on up to 4096 separate strands.

occurs at meiotic prophase. As the cells increase in size so the chromosomes repeatedly divide but *without separation of the products of replication,* so that a very thick polytene (literally, many stranded) chromosome, consisting of up to 4096 individual strands of chromatin, is gradually built up (figure 4.11). Each chromatin strand is 20–30 nm in diameter and is continuous throughout the euchromatic regions of the chromosome. The polytene chromosomes found in the salivary gland cells are among the largest chromosomes known and may be up to 2 mm long, over 100 times the length of a somatic metaphase chromosome, and clearly visible with a hand lens (figure 4.12).

Figure 4.12 A polytene chromosome. Chromosome IV from a salivary gland cell of *Drosophila melanogaster.* This is the smallest of the *Drosophila* chromosomes and is only about 50 μm long; it contains about 140 bands.

The effect of polytenisation has been to increase the detailed structure of the chromosomes by several orders of magnitude, so that any minute differences in lateral structure of the basic chromatin fibres are enormously magnified. When the many hundreds of identical chromomeres (genes?) lie side by side in this way they form a well-defined *band.* These bands represent regions where the chromatin fibres are more tightly packed and they vary so much in their size and appearance that even a very short segment of a chromosome has a specific and identifiable pattern of banding. Thus any change in the structure of a chromosome is reflected in a change in the pattern of banding (see chapter 10), making it possible to associate particular genes with particular chromosome bands. In no instance has it been shown that more than one gene or group of closely related genes lies in any particular band, and there is a growing body of genetic evidence to suggest that each band corresponds to just one gene or to a group of closely related genes. Over 5000 bands have been identified in *Drosophila*–whether this number of genes is adequate for an organism of this degree of complexity remains to be seen; by way of comparison it is interesting to note that if the average gene is about 1000 nucleotide pairs long then the chromosome of *E. coli* could accommodate about 3000 genes.

Summary of Key Points

(1) In addition to DNA the eucaryotic chromosome contains protein and RNA; the most important proteins are the histones, which probably assist in packaging the DNA and in controlling gene action.

(2) During most of the cell cycle the chromosomes are not visible as such and are spun out into long thin filaments beyond the resolution of the light microscope; during this stage of the cell cycle the DNA replicates.

(3) The cells divide by the process of mitosis. During mitosis the chromosomes contract and become visible and are seen to be divided into two chromatids. One chromatid from each chromosome passes into each daughter cell. This regular segregation of the chromatids is assisted by a protein structure, the mitotic spindle.

(4) During gametogenesis a special type of cell division, meiosis, ensures that each gamete contains only one representative of each chromosome pair. This is achieved by there being two divisions of the cell accompanied by only one division of the chromosomes.

(5) The chromosomes, like molecules of DNA, replicate semi-conservatively.

(6) It is thought that the eucaryotic chromosome consists of one very long thread of DNA folded back and forth on itself and encased in a protein coat.

(7) The DNA from eucaryotic chromosomes replicates in both directions from a number of initiation points.

(8) Special technique described is the autoradiography of eucaryotic chromosomes.

5 Genetic Analysis in Higher Organisms

Busy, curious, thirsty fly
Drink with me, and drink as I.
William Oldys (1696-1761)

Much of the difficulty experienced in handling genetic data occurs because of an inadequate understanding of the basic principles of heredity. The importance of mastering genetic analysis cannot be stressed too much, for it is the basic tool of all geneticists from the plant breeder to the molecular biologist. We will start by considering the inheritance of a single character difference and progress to more complicated situations where several character differences are being inherited together, using the fruit fly, *Drosophila melanogaster,* and maize, *Zea mays,* as our principal experimental organisms.

Drosophila

Drosophila, perhaps the most famous of all genetic 'guinea pigs' as it has been intensively studied for over sixty years, is a very convenient laboratory organism as it is easily cultured, has a rapid generation time (twelve days), and each fertilised female can produce up to 400-500 progeny (figure 5.1); thus relatively large numbers of progeny can easily be handled in a short time (compare this with mice, which have a generation time of two months and an average litter size of five). It has the further advantages of having only four pairs of chromosomes, one pair of which is extremely small and carries relatively few genes, and of having giant polytene chromosomes (page 61) in the salivary glands, making it possible to correlate genetic and cytological studies.

Maize

Maize has an even longer history as a genetic organism, for both Hugo de Vries and Carl Correns observed Mendelian ratios in it in their experiments leading to the rediscovery of Mendelism, and as early as 1912, linkage in maize was observed by G. N. Collins. Very soon after the rediscovery of Mendel's work it was realised that his principles of heredity had very far reaching consequences in practical plant breeding, and because of the great economic importance of improving the quality and quantity of maize crops it was inevitable that considerable attention would focus on maize genetics. One of the most far reaching developments in the applied biology of its time was the outcome of this research, the development of hybrid corn. Among the many advantages that make maize a favourable organism for genetic study are, first, that separate male

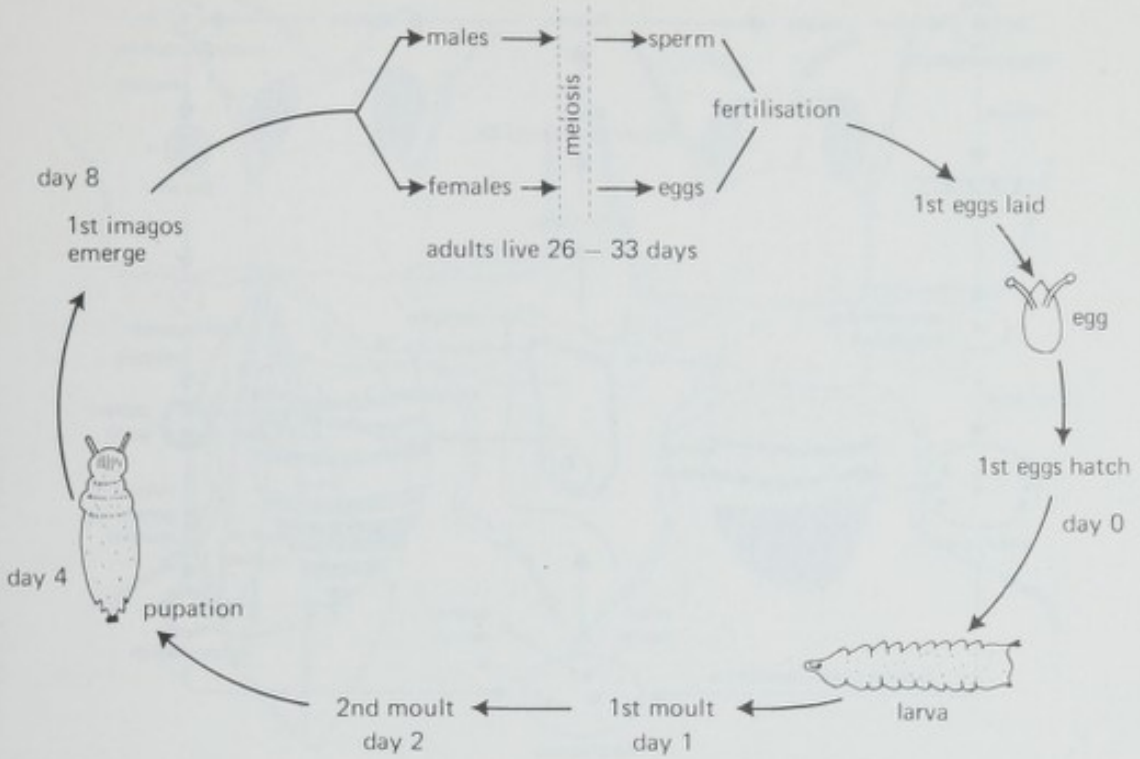


Figure 5.1 The life cycle of *Drosophila melanogaster*. In the laboratory *Drosophila* is bred by placing fertilised females in a milk bottle containing about 2 in. depth of a medium made from maize meal, treacle and agar fibre. The eggs are laid on this medium and after hatching the larvae burrow into and feed on it; after about four days the larvae crawl up a filter paper stuck into the medium and pupate.

and female flowers occur on the same plant; thus controlled pollinations are easily effected by tying a paper bag over each female inflorescence shortly before the silks (the styles) develop, so preventing pollination by stray pollen, and at the right time applying to the silks pollen collected from the tassels (male inflorescences) of the plant selected for use as the male parent. There is no need for the tedious emasculation that is necessary when both stamens and pistils occur in the same flower. Second, many thousands of kernels (seeds) can be obtained with relatively little expense or trouble; these kernels are large and seed characters can easily be scored. Third, there is a very large number of mutant characters affecting all aspects of plant form and function. Finally, each of the ten chromosome pairs is morphologically recognisable.

The life cycle of maize (figure 5.2) is that of a typical angiosperm. When fertilisation occurs one sperm nucleus (n, haploid) fuses with an egg nucleus (n) to form the zygote or embryo (2n), and the second sperm nucleus fuses with the two polar nuclei to form a triploid (3n) nucleus, which by a series of mitotic divisions produces the endosperm, the food reserve of the seed. The whole seed is protected by a layer of maternal tissue, the *pericarp*. A point to note is that the two sperm nuclei on the one hand, and the egg and polar nuclei on the other hand, are genetically identical.

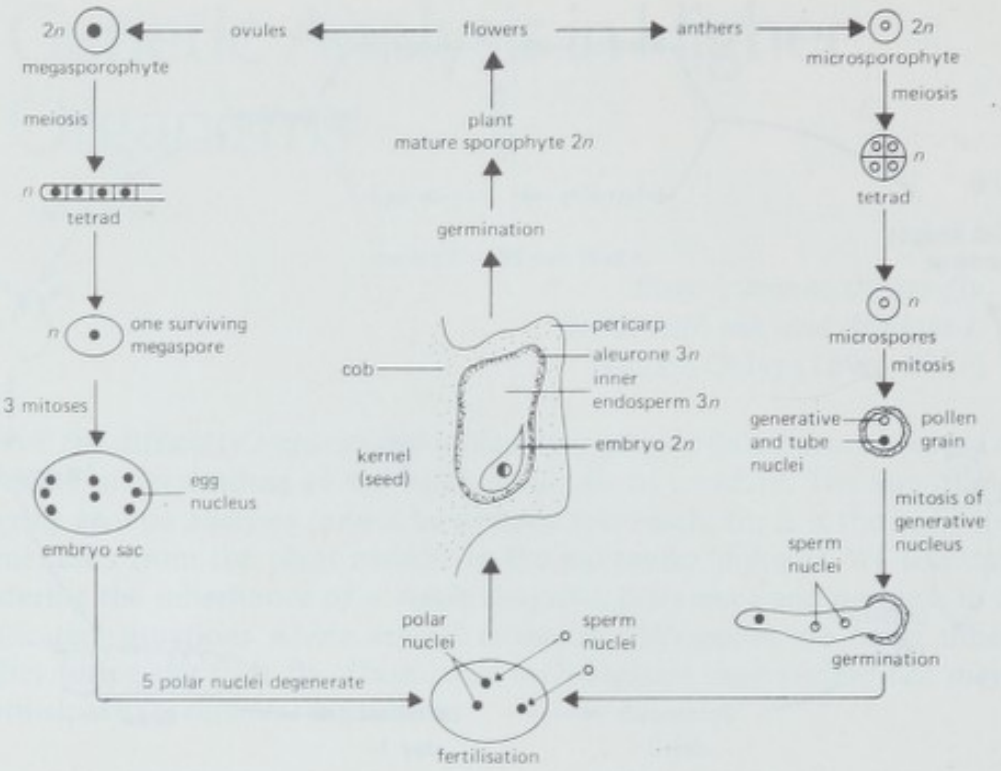


Figure 5.2 The life cycle of maize. In the male gametophyte the two sperm nuclei and the tube nucleus are all derived from a single microspore nucleus by mitosis, and so are genetically identical. Likewise, the polar and egg nuclei of the female gametophyte are genetically identical. At fertilisation one sperm nucleus fuses with the egg nucleus to form the zygote while the other sperm nucleus fuses with the two remaining polar nuclei and eventually produces the triploid endosperm.

Independent Segregation

One laboratory stock of *Drosophila melanogaster* has very reduced or *vestigial* wings (figure 5.3). Flies of this phenotype are homozygous for a recessive gene located on chromosome II; this recessive gene is given the symbol *vg* and the genotype of the vestigial winged flies is written *vg*/*vg* or *vg vg*. The normal or wild type strain is homozygous for the wild type allele, usually designated vg^+ or, if no confusion is likely, just as +. Both the vestigial and the wild-type strains are true breeding and when bred among themselves produce all vestigial or all wild type flies respectively.

What happens if we *cross* or mate a vestigial winged fly with a wild type fly? The formula for this cross is written

$$vg/vg \times vg^+/vg^+ \qquad \text{P}$$

with the genotype of the female parent on the left and of the male on the right. P stands for the parental generation. Each female gamete receives one member of the chromosome II pair, but since both homologues carry the *vg* allele all the gametes will be *vg*. Likewise, every male gamete will bear a vg^+ allele. The progeny of the first filial or F_1 generation are the outcome of fusions between

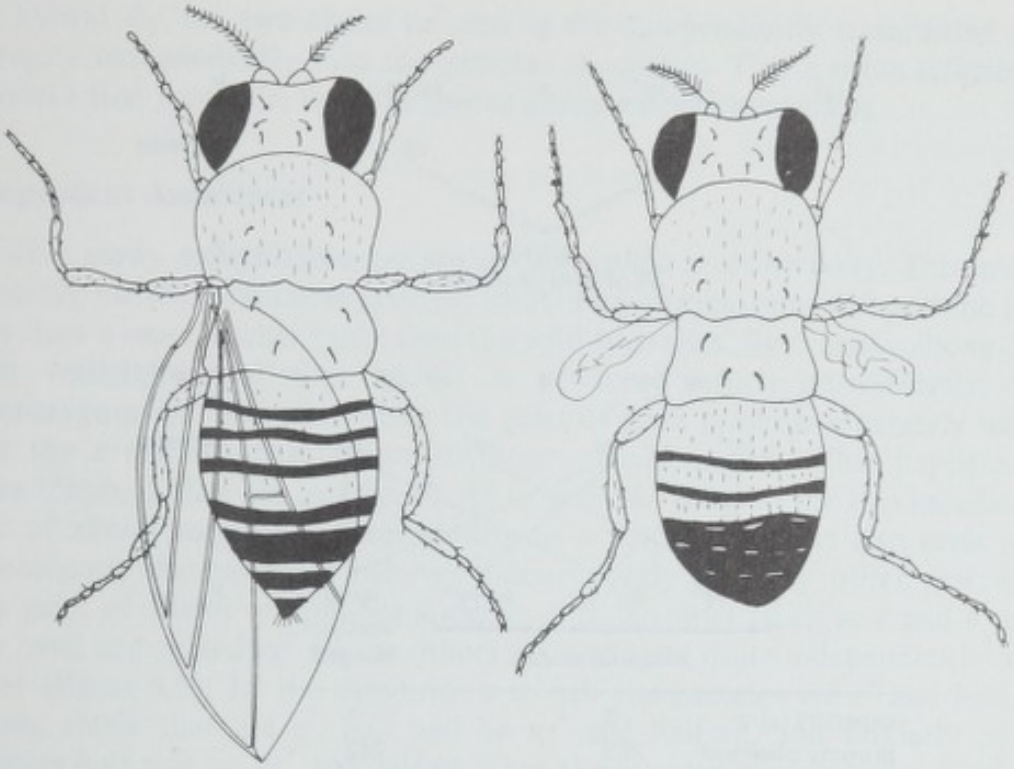


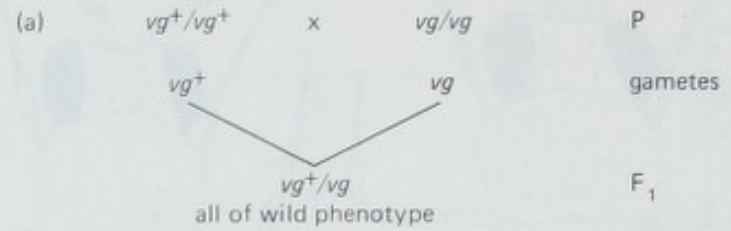
Figure 5.3 *Drosophila melanogaster.* A normal female (left) and a male with vestigial wings (right). The female is larger and the abdomen more pointed and darker in colour. For clarity, one wing of the normal female has been omitted.

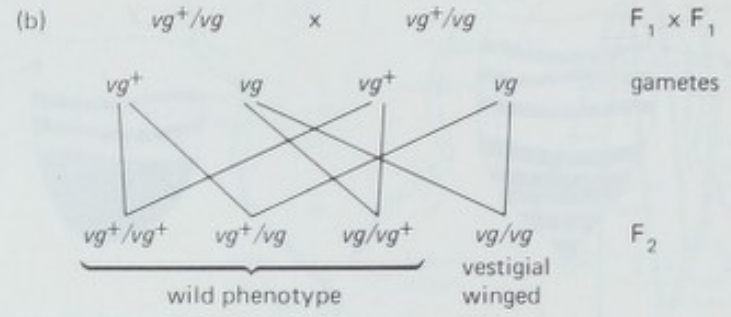
these two types of gamete and will be uniformly heterozygous; every fly will be vg/vg^+ and, because vg^+ is dominant over vg, will have a wild phenotype (figure 5.4). These F_1 flies can now be crossed among themselves to produce the second, or F_2, generation. The F_1 flies are all vg/vg^+ heterozygotes and, because of the regularity of meiosis, half the gametes they produce are vg and half are vg^+. When these male and female gametes unite randomly four progeny genotypes are produced in statistically equal numbers (figure 5.4); three out of every four progeny (vg^+/vg^+, vg^+/vg and vg/vg^+) have the wild phenotype and one (vg/vg) the mutant phenotype, so that we expect a 3:1 ratio among the progeny phenotypes.

Instead of selfing the F_1, F_1 females could be *backcrossed* to the male homozygous recessive parent (or *vice versa*). The F_1 parent will again produce ½ vg and ½ vg^+ gametes but all of these will be fertilised by a vg gamete from the other parent and there will be an expected ratio of 1:1 among the F_2 flies.

We can now see the first use of the genetic cross. Both vg^+/vg^+ and vg^+/vg flies have identical wild phenotypes and cannot be distinguished by eye, but by making a *test cross,* crossing a fly of unknown genotype with a vg/vg fly, we can ascertain whether it is vg^+/vg^+ (when only wild-type progeny are recovered) or vg^+/vg (when there is a 1:1 ratio of wild-type and vestigial-winged progeny).

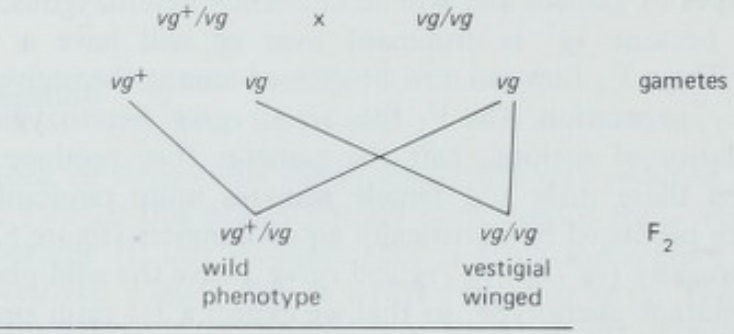
The reappearance of the recessive character in the F_2 generation shows that the recessive vg gene was neither lost or changed in the hybrid F_1, and that in





expected ratio	3	1
progeny observed	763	242
progeny expected	753.75	251.25
total progeny	1005	

(c) or backcrossing the F_1



expected ratio	1	1
progeny observed	1075	1243
progeny expected	1159	1159
total progeny	2318	

Figure 5.4 Independent segregation of a pair of alleles in *Drosophila*. Showing (a) the parental cross and how (b) a cross between two F_1 hybrids produces a 3 : 1 ratio of F_2 progeny phenotypes and (c) the backcross produces a 1:1 ratio of progeny phenotypes. Also shown are the numbers of progeny flies observed in these crosses and the numbers expected assuming these ratios. Note that because of the laws of chance, exact ratios will only very rarely be observed and that the greater the number of progeny scored the better will be the agreement between the observed and expected numbers.

the hybrid F_1 the two alleles vg^+ and vg are independently transmitted and so segregate independently into the gametes at meiosis. This is often referred to as Mendel's first principle, the *principle of independent segregation.*

Independent Assortment

Another easily recognisable mutant of *Drosophila* is *ebony body.* This mutant is homozygous for a recessive gene (e) located on chromosome III, and the mutant flies have a much darker body than the wild type flies. By crossing ebony-bodied with vestigial-winged flies an F_1 is produced which is uniformly doubly heterozygous e/e^+ vg/vg^+ (writing the pairs of gene symbols separately indicates that the e and vg genes are on different chromosomes). What happens when these dihybrid flies are interbred? At meiosis not only do the two members of a pair of alleles segregate independently in a 1:1 fashion but also each pair of homologous chromosomes behaves independently of every other pair, so that two pairs of alleles on non-homologous chromosomes (such as e and e^+ on the one hand and vg and vg^+ on the other) will segregate quite independently of each other (figure 5.5). In the dihybrid, one half the gametes are e^+ and half are e; among those that are e^+ half will be vg^+ and half vg, and similarly of the e gametes half will be vg^+ and half vg. Thus the four types of gamete, e^+ vg^+, e^+ vg, e vg^+ and e vg, are produced in statistically equal numbers.

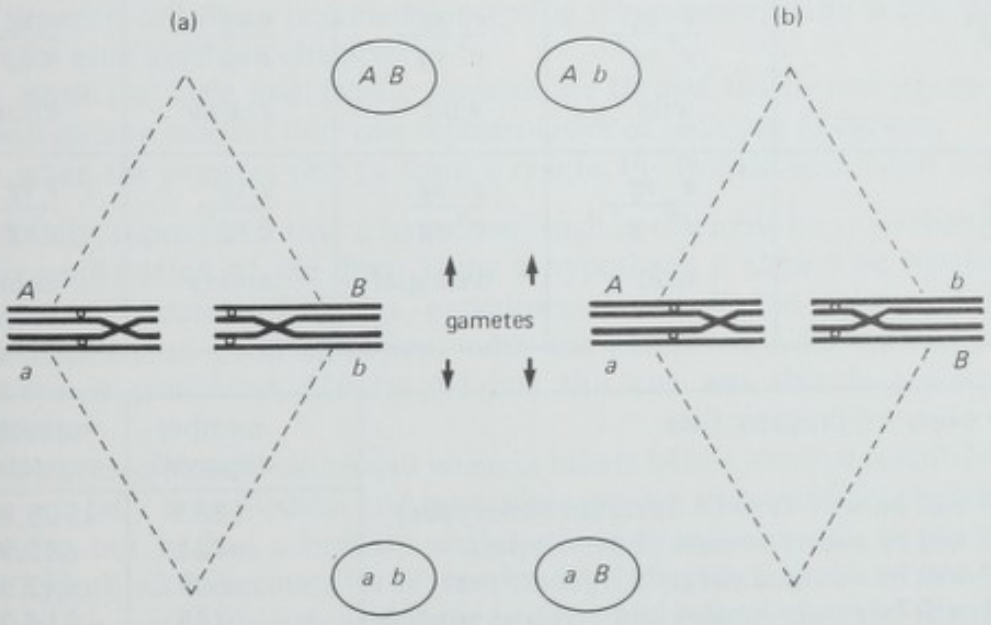


Figure 5.5 Independent assortment. A–a and B–b are two pairs of genes located on different chromosomes. At meiosis the two pairs of chromosomes (bivalents) orientate at random on the metaphase plate so that the two arrangements (a) and (b) occur at equal frequency and equal numbers of the four types of gamete will be produced.

When the $e^+/e\ vg^+/vg$ flies are interbred, the four types of male gamete and the four types of female gamete will unite at random, that is, in all possible combinations. The sixteen possible gametic combinations (figure 5.6) produce four phenotypes distributed among nine different genotypes. When the pairs of alleles are segregating independently we expect a 9:3:3:1 ratio in the F_2. In just

P	$e/e \quad vg^+/vg^+$	$\times\ e^+/e^+ \quad vg/vg$
F_1	$vg^+/vg \quad e^+/e$	

When F_1 flies are interbred

female gametes	male gametes			
	$e^+\ vg^+$	$e^+\ vg$	$e\ vg^+$	$e\ vg$
$e^+\ vg^+$	$\frac{e^+\ vg^+}{e^+\ vg^+}$ wild	$\frac{e^+\ vg^+}{e^+\ vg}$ wild	$\frac{e^+\ vg^+}{e\ \ vg^+}$ wild	$\frac{e^+\ vg^+}{e\ \ vg}$ wild
$e^+\ vg$	$\frac{e^+\ vg}{e^+\ vg^+}$ wild	$\frac{e^+\ vg}{e^+\ vg}$ vestigial	$\frac{e^+\ vg}{e\ \ vg^+}$ wild	$\frac{e^+\ vg}{e\ \ vg}$ vestigial
$e\ vg^+$	$\frac{e\ \ vg^+}{e^+\ vg^+}$ wild	$\frac{e\ \ vg^+}{e^+\ vg}$ wild	$\frac{e\ vg^+}{e\ vg^+}$ ebony	$\frac{e\ vg^+}{e\ vg}$ ebony
$e\ vg$	$\frac{e\ \ vg}{e^+\ vg^+}$ wild	$\frac{e\ \ vg}{e^+\ vg}$ vestigial	$\frac{e\ vg}{e\ vg^+}$ ebony	$\frac{e\ vg}{e\ vg}$ ebony vestigial

For every 16 progeny flies	number observed	number expected
9 will be wild type (4 different genotypes)	1853	1928.82
3 will be ebony bodied (2 genotypes)	721	642.94
3 will be vestigial winged (2 genotypes)	607	642.94
1 will be ebony bodied and vestigial winged	248	214.31
	Totals 3429	3429

Figure 5.6 Independent assortment of two pairs of alleles in *Drosophila*. When the four types of male and four types of female gamete unite at random the sixteen possible genotypes are distributed among four different phenotypes in a 9:3:3:1 ratio.

the same way the backcross $e^+/e\ vg^+/vg \times e/e\ vg/vg$ will produce the same four phenotypes but in a 1:1:1:1 ratio. This is the *principle of independent assortment*, often referred to as Mendel's second principle.

Knowing how the chromosomes behave at meiosis it is not difficult to predict how the genes will segregate at gametogenesis, but Mendel, when he did his experiments with peas, knew nothing about meiosis or the significance of chromosomes. Yet from the results of simple crosses with peas he was able to make deductions that were years ahead of their time. It is instructive to summarise Mendel's own conclusions and to see how exactly they agree with the predictions we made based on our knowledge of chromosome behaviour. He observed that:

(1) in the F_1 only one of the alternative characters that distinguished the parental variety appears, the dominant character;

(2) in the F_2 both alternative characters appear among the progeny, the dominant character being three times more frequent than the recessive character;

(3) when the F_2 seed was grown and the flowers self-fertilised, all of the seed showing the recessive character and one third of the seed showing the dominant character bred true—the other two-thirds of the seed with the dominant character behaved as did the F_1 hybrid.

He was able to deduce:

(1) each given characteristic is determined by a pair of particulate factors;

(2) each factor is transmitted from parent to offspring as a virtually unchanging unit;

(3) in each cell of an organism (excepting the gametes) there is one pair of factors for each inherited character;

(4) when the male and female gametes are formed the factors segregate so that each gamete receives only one representative of each pair of factors;

(5) when the gametes fuse to form a zygote, the factors again occur in pairs.

Mendel fully appreciated that a hypothesis such as this must be more than just a possible explanation of the data. Using a hypothesis it should be possible to predict the outcome of future experiments, and if the results of these experiments are in good agreement with the predictions, the validity of the hypothesis is confirmed. Mendel did just this and was able to confirm his hypothesis.

How is it that Mendel succeeded whereas others before him had failed? Firstly, he was not only a methodical and meticulous worker who carefully recorded all his results, but he had a brilliant analytical mind and was able to propose a simple hypothesis to account for his results. Secondly, he chose his experimental material with care. Although peas are normally self-fertilising, Mendel found that he could cross-fertilise the plant by removing the stamens before self-fertilisation occurred and dusting the stigmas with pollen from a different plant. He was also careful to use only pairs of true breeding and sharply contrasting characters such as round versus wrinkled and green versus yellow seeds (the use of seed characters in plant breeding is particularly advantageous as the seed formed on

the parental plant *is* the F_1 generation, so that the characters can be scored without having to grow the seed); he carefully avoided characters such as seed size and leaf shape which show a continuous range of phenotypes (i.e. continuous variation). Another of the reasons for his success was that he examined the inheritance of only one or two such characters at a time. In one respect Mendel was fortunate as the genes controlling the several character differences he studied are on different chromosomes; we cannot even guess what conclusions he might have made had the genes been on the same chromosome and *linked.*

In *Drosophila* several hundreds of gene loci have been identified (a locus is the 'location' of a gene along a chromosome) and since there are only four pairs of chromosomes each chromosome must carry very many genes; it is when we study loci on the same chromosome that we sometimes observe the phenomenon of linkage.

Linkage

Shortly following the rediscovery of Mendel's work several workers, among them William Bateson and R. C. Punnett, observed deviations from this principle of independent assortment; they found that there was sometimes a tendency for the two alleles inherited from one parent to remain together during the formation of the gametes. In a like way the pair of alleles inherited from the other parent would also tend to remain together.

If *A, a* and *B, b* are two pairs of alleles, a cross between *AABB* and *aabb* will produce uniform *AaBb* progeny and when there is independent assortment the backcross *AaBb* × *aabb* will produce equal numbers of *AB, Ab, aB* and *ab* progeny; in the exceptions observed by Bateson and Punnett there were excesses of the two parental combinations *AB* and *ab.* They called this phenomenon linkage, although they failed to recognise its significance.

As the chromosome is the unit of segregation at meiosis, genes which are on the same chromosome may not assort independently. We can define linkage as the tendency for genes on the same chromosome to remain together when they enter the gametes, so that the parental character combinations appear more frequently among the progeny than the non-parental combinations. If the chromosome is the unit of segregation why do not the genes along it always remain associated? Why, in the preceding example, do we not recover only *AB* and *ab* phenotypes among the progeny, and how have the *Ab* and *aB* non-parental or *recombinant* combinations arisen.

You will recollect that at the prophase of meiosis (chapter 4) the two members of a pair of homologous chromosomes pair along their length, split into chromatids (figure 5.7a(1)) and undergo crossing-over or recombination. Recombination occurs just as if two non-sister chromatids break at precisely corresponding points and rejoin in a new combination (figure 5.7a(2)) with the result that genetic material is exchanged between these two non-sister chromatids. Two of the four products of meiosis (figure 5.7a(3)) contain parental and two contain recombinant chromatids. The chiasmata observed at

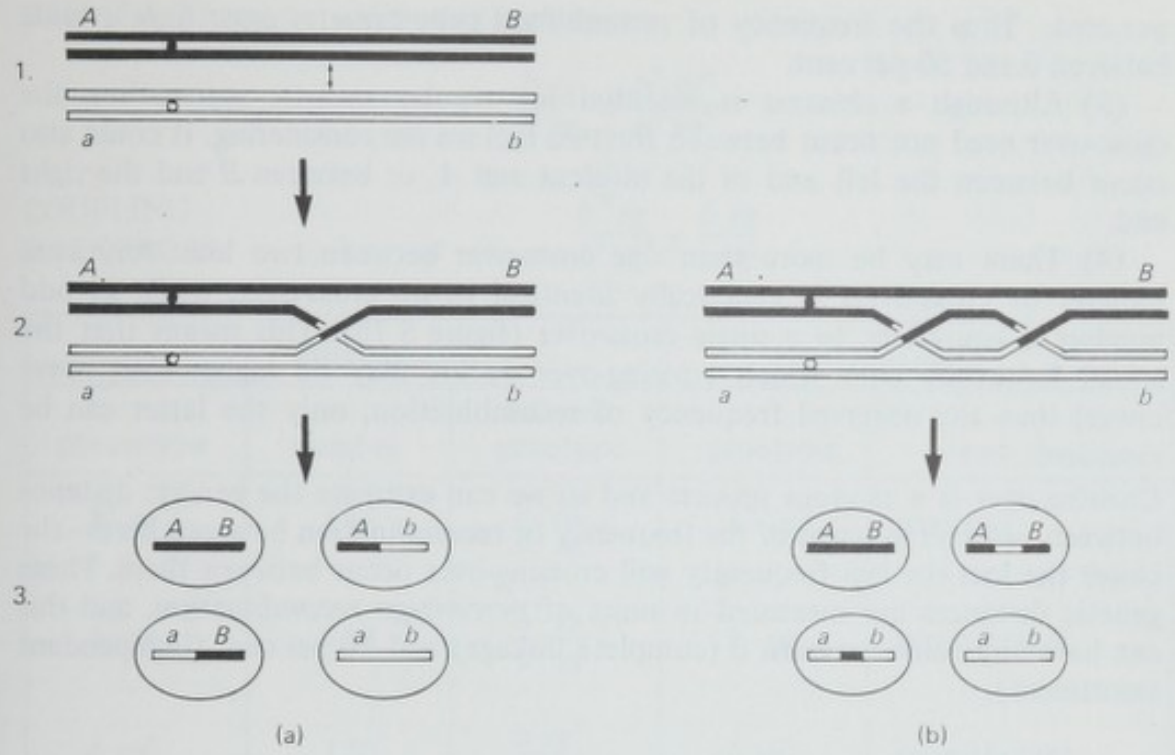


Figure 5.7 Recombination. (a) At meiosis two homologous chromosomes pair along their length and divide into chromatids (1). Breaks occur at homologous points on two non-sister chromatids (indicated by the small arrows) which then unite in a new combination (2); the resulting chiasma is necessary to ensure the regular segregation of the pairs of chromatids at anaphase I. As a consequence of crossing-over two of the four products of meiosis (3) show recombination for markers on opposite sides of the breaks. (b) When two cross-overs occur in the same interval (between *A* and *B*) the outcome is the same as when no crossing-over occurs. Similarly, any odd number of cross-overs has the same outcome as a single cross-over.

late prophase are essential if meiosis is to proceed normally and it seems probably that they are the visible outcomes of crossing-over. We do not yet understand the details of the recombinational process but a possible molecular model is described in chapter 16.

Note carefully the following points:

(1) Because the breaks occur at homologous positions on two non-sister chromatids recombination is an exactly reciprocal process. For every *Ab* gamete there must also be a *aB* gamete.

(2) Each cross-over involves only two of the four chromatids, so that of the four products of meiosis two are recombinant and two are parental types. Consequently, no matter how frequently crossing-over occurs between a given pair of loci, the proportion of recombinant type gametes can *never* exceed 50 per cent. When crossing-over occurs so frequently that 50 per cent of the gametes are recombinant and 50 per cent are parental types, the genes will assort independently and so appear to be unlinked. On the other hand, the loci may be very close together on the chromosome so that crossing-over rarely occurs between them, and the proportion of recombinant type gametes will approach 0

per cent. Thus the frequency of recombinant type gametes *must* have a value between 0 and 50 per cent.

(3) Although a chiasma is essential for regular meiotic segregation, the cross-over need not occur between the two loci we are considering. It could also occur between the left end of the bivalent and *A*, or between *B* and the right end.

(4) There may be more than one cross-over between two loci. Any even number of cross-overs is genetically identical to no cross-over, while an odd number is equivalent to a single cross-over (figure 5.7b). This means that the actual frequency with which crossing-over occurs may be higher (but never lower) than the observed frequency of recombination; only the latter can be measured.

Crossing-over is a random process and so we can estimate the genetic distance between two loci in terms of the frequency of recombination between them—the closer the loci the less frequently will crossing-over occur between them. These genetic distances are measured in units of *percentage recombination*, and this can have any value between 0 (complete linkage) and 50 per cent (independent assortment).

Two-point Linkage

In *Drosophila* the *b* and *vg* loci are both on chromosome II; *bb* flies ('black') have very dark bodies. Let us consider a cross where a double heterozygote, produced by crossing a wild-type fly with a double homozygous recessive fly, is backcrossed to a double homozygous recessive male (table 5.1).

If there is independent assortment we expect a 1:1:1:1 ratio of b^+ vg^+, b^+ vg, b vg^+ and b vg progeny phenotypes, but if there is linkage we expect there to be an excess of the parental b^+ vg^+ and b vg combinations. Note how the formula for the genotype is written when linked genes are involved. The genes on one chromosome are shown above the unbroken horizontal line while those on the other homologue are shown below this line; the formula not only tells us that these loci are linked, but also which are the parental combinations. When the two dominant genes are on one chromosome and the two recessive genes on the other they are said to be in the *coupling* phase.

Table 5.1 shows the results of this coupling backcross. Observe that the two types of recombinant gametes (b^+ vg and b vg^+) occur at the same frequency; this is expected, as for every b^+ vg gamete formed at meiosis there is also a b vg^+ gamete. Likewise, the parental type gametes (b^+ vg^+ and b vg) occur at the same frequency. In this backcross each progeny fly receives only the recessive alleles *b* and *vg* from its father, so that from its phenotype it is possible to deduce the genotype of the fly and hence the alleles it received through the maternal gamete.

These data illustrate the advantage of using the double recessive homozygote as one parent; if the second parent was also heterozygous at both loci then recombination in *both* parents would have to taken into account and the analysis becomes more complex. However, this problem does not arise with

Table 5.1 Linkage in a coupling backcross

$$\frac{b\ vg}{b\ vg} \times \frac{b^+\ vg^+}{b^+\ vg^+}$$

COUPLING BACKCROSS

$$\frac{b^+\ vg}{b^+\ vg^+} \times \frac{b\ vg}{b\ vg}$$

Progeny			Female gamete	
phenotype	number	inferred genotype	genotype	type
$b^+\ vg^+$	586	$\frac{b^+\ vg^+}{b\ vg}$	$b^+\ vg^+$	parental
$b^+\ vg$	112	$\frac{b^+\ vg}{b\ vg}$	$b^+\ vg$	recombinant
$b\ vg^+$	120	$\frac{b\ vg^+}{b\ vg}$	$b\ vg^+$	recombinant
$b\ vg$	465	$\frac{b\ vg}{b\ vg}$	$b\ vg$	parental
Total	1283			

$$\text{percentage recombination} = \frac{\text{total recombinant gametes}}{\text{total gametes}} \times 100$$

$$= \frac{112 + 120}{1283} \times 100 = 18.08 \text{ per cent}$$

i.e. among the 1283 viable female gametes that contributed to the progeny, 232, or 18.08 per cent were recombinant between *b* and *vg*.

Drosophila as for some unexplained reason crossing-over and recombination only occur in females; had the heterozygous flies been used as the male parent in the coupling backcross

$$\frac{b\ vg}{b\ vg} \times \frac{b^+\ vg^+}{b\ \ vg}$$

only parental type ($b^+\ vg^+$ and $b\ vg$) progeny phenotypes would have been recovered.

Linkage is not the only explanation of these data. They could also be explained by assuming that there is independent assortment, so that the four types of gamete are produced in equal numbers, but that the $b^+ vg$ and $b\ vg^+$ gametes have a comparatively low viability; this situation would also give rise to an excess of the so-called parental types. Critical evidence that linkage is involved comes from a comparison of the coupling backcross data (table 5.1) with the data from the corresponding *repulsion* backcross (table 5.2). In the

Table 5.2 Linkage in a repulsion backcross

$$\frac{b\ vg^+}{b\ vg^+} \times \frac{b^+ vg}{b^+ vg}$$

REPULSION BACKCROSS

$$\frac{b^+ vg^+}{b^+ vg} \times \frac{b\ vg}{b\ vg}$$

Progeny			Female gametes	
phenotype	number	inferred genotype	genotype	type
$b^+ vg^+$	338	$\frac{b^+ vg^+}{b\ vg}$	$b^+ vg^+$	recombinant
$b^+ vg$	1552	$\frac{b^+ vg}{b\ vg}$	$b^+ vg$	parental
$b\ vg^+$	1315	$\frac{b\ vg^+}{b\ vg}$	$b\ vg^+$	parental
$b\ vg$	294	$\frac{b\ vg}{b\ vg}$	$b\ vg$	recombinant
Total	3499			

$$\text{percentage recombination} = \frac{338 + 294}{2499} \times 100$$

$$= 18.06 \text{ per cent}$$

repulsion backcross doubly heterozygous flies, obtained by crossing black bodied flies with vestigial winged flies, are backcrossed to doubly homozygous recessive male flies. The dominant alleles in these heterozygotes are on different chromosomes and they are said to be in the repulsion phase. Note that in this cross there is an excess of $b^+ vg$ and $b\ vg^+$ gametes; if differential viability were

the explanation of the coupling backcross data, we would expect there to be no difference between the data from the two backcrosses. The percentage recombination values obtained from the two sets of data are essentially the same; thus percentage recombination is a property of the two genes recombining and is independent of the way in which the alleles are arranged on the chromosomes.

Three-Point Linkage

We have seen that the farther apart two loci are on a chromosome, the greater is the chance that recombination will occur between them and so the greater the percentage recombination value. This makes it possible to construct *linkage maps*, genetic maps of the chromosomes, showing the sequence of the loci along the chromosomes and their relative distances apart in terms of percentage recombination. Since the percentage recombination values are approximately additive, a simple linkage map can be constructed from the data of several independent two-point crosses (figure 5.8) but more often a three-point cross is used. Look carefully and you will realise that the three-point cross is rather more than the sum of the three separate two-point crosses.

The three-point cross is best illustrated by calculating the linkage relationships from a set of backcross data. Given the following progeny phenotypes in *D. melanogaster* (*cn*/*cn* flies have cinnabar coloured eyes instead of red eyes) let us construct the linkage map.

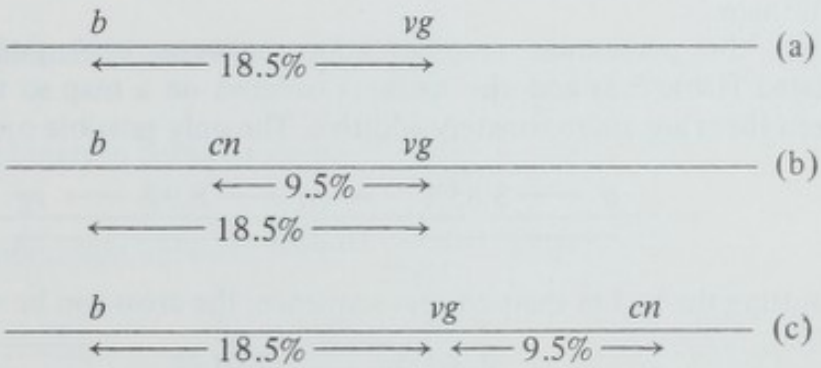


Figure 5.8 Constructing a linkage map. The *b* and *vg* loci in *Drosophila* are both on chromosome II with about 18.5 per cent recombination between them. In another experiment *cn* (*cn*/*cn* flies have cinnabar coloured eyes instead of red) is found to show 9.5 per cent recombination with *vg*; *cn*, could be located *either* between *b* and *vg* (b), when there should be about 9 per cent recombination between *b* and *cn*, *or* beyond *vg* (c), when there should be about 28 per cent recombination between *b* and *cn*. A further experiment establishes that the percentage recombination between *b* and *cn* is about 9 per cent; hence *cn* is located mid-way between *b* and *vg* (b).

The first requirement in assessing any linkage data is to recognise the parental and recombinant gene combinations. This is only possible when we know how

$cn^+ \ b^+ \ vg^+$	92	$cn^+ \ b^+ \ vg$	6	
$cn \ b \ vg$	70	$cn \ b \ vg^+$	9	Total 2000
$cn \ b^+ \ vg$	792	$cn \ b^+ \ vg^+$	86	
$cn^+ \ b \ vg^+$	868	$cn^+ \ b \ vg$	77	

the genes are arranged in the heterozygous parent but this is easily deduced if we remember that for any pair of genes there must *always* be more parental than recombinant types. In both the two-point backcrosses (tables 5.1 and 5.2) the parental types are in excess and inspection of the data would, if it were not known, indicate whether the markers were in coupling or repulsion. Similarly, in the three-point backcross data the $cn^+ \ b \ vg^+$ and $cn \ b^+ \ vg$ phenotypes are by far the most frequent and so are probably the parental combinations (if we make an incorrect deduction we will get a percentage recombination value that is over 50 per cent, which we know must be wrong). The cross can now be written

$$\left(\frac{cn^+ \ b \ vg^+}{cn \ b^+ \ vg}\right) \times \left(\frac{cn \ b \ vg}{cn \ b \ vg}\right)$$

the brackets indicating that we do not yet know the relative order of the genes within them.

Next, the percentage recombination between *each pair* of markers is calculated (table 5.3) and the markers ordered on a map so that the distances between them are approximately additive. The only possible order is

$$\underline{b \longleftarrow 8.85\% \longrightarrow cn \longleftarrow 8.9\% \longrightarrow vg}$$
$$\longleftarrow 16.25\% \longrightarrow$$

and, putting the loci in their correct sequence, the cross can be written

$$\frac{b \ cn^+ \ vg^+}{b^+ \ cn \ vg} \times \frac{b \ cn \ vg}{b \ cn \ vg}$$

Observe that there is an apparent discrepancy, as $8.85 + 8.9 \neq 16.25$. The reason for this will be clear if we consider the origin of each class of gamete. The $b^+ \ cn \ vg$ and $b \ cn^+ \ vg^+$ gametes are non-recombinant both between *b* and *cn* and between *cn* and *vg* (figure 5.9). The $b^+ \ cn^+ \ vg^+$ and *b cn vg* gametes have arisen by a single recombination event occurring between *b* and *cn*, while the $b^+ \ cn \ vg^+$ and $b \ cn^+ \ vg$ gametes have arisen by recombination in the interval between *cn* and *vg*. The $b^+ \ cn^+ \ vg$ and $b \ cn \ vg^+$ gametes are the result of recombination in both the interval between *b* and *cn* and between *cn* and *vg*. Examination of table 5.3 will reveal that the last two classes of gamete have been scored as recombinant between *b* and *cn*, as recombinant between *cn* and *vg*, *but* as non-recombinant between *b* and *vg*. Clearly they cannot be non-recombinant and in fact they are

Table 5.3 The calculation of linkage in a three-point cross

$$\frac{cn^+\ b\ vg^+}{cn\ \ b^+\ vg} \times \frac{cn\ b\ vg}{cn\ b\ vg}$$

progeny phenotype	number observed	whether recombinant between		
		cn–b	*b–vg*	*cn–vg*
$cn^+\ b^+\ vg^+$	92	yes	yes	
$cn\ \ b\ \ vg$	70	yes	yes	
$cn\ \ b^+\ vg$	792			
$cn^+\ b\ \ vg^+$	868			
$cn^+\ b^+\ vg$	6	yes		yes
$cn\ \ b\ \ vg^+$	9	yes		yes
$cn\ \ b^+\ vg^+$	86		yes	yes
$cn^+\ b\ \ vg$	77		yes	yes
	2000	177	325	178

percentage recombination between *b–vg* $= \frac{325}{2000} \times 100 = 16.25$

percentage recombination between *cn–vg* $= \frac{178}{2000} \times 100 = 8.9$

percentage recombination between *b–cn* $= \frac{177}{2000} \times 100 = 8.85$

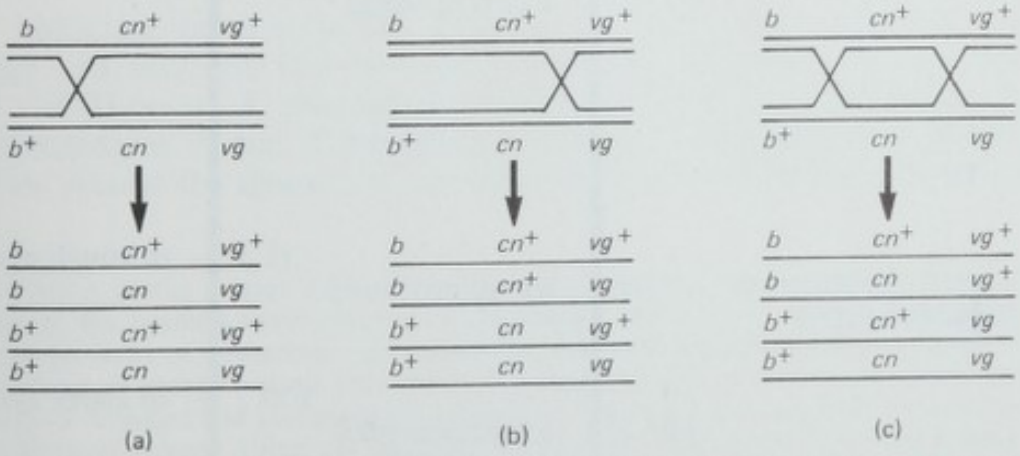
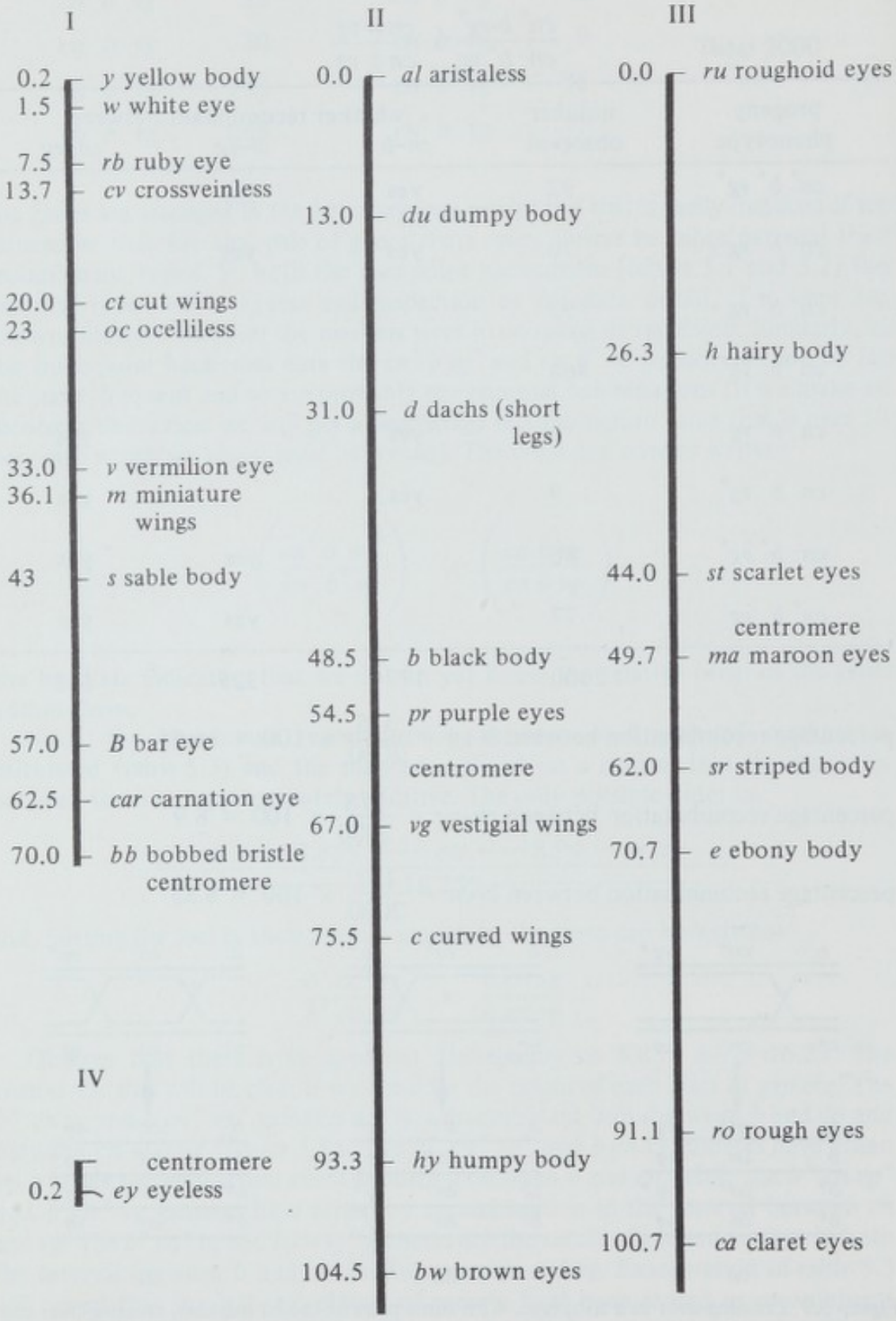


Figure 5.9 Crossing-over in a trihybrid. With three pairs of linked markers, crossing-over can occur between the left-hand pairs of markers (a), between the right-hand pairs (b) or between both pairs (c) and will produce the four types of gamete shown.


I
0.2 y yellow body
1.5 w white eye
7.5 rb ruby eye
13.7 cv crossveinless
20.0 ct cut wings
23 oc ocelliless
33.0 v vermilion eye
36.1 m miniature wings
43 s sable body
57.0 B bar eye
62.5 car carnation eye
70.0 bb bobbed bristle
centromere
II
0.0 al aristaless
13.0 du dumpy body
31.0 d dachs (short legs)
48.5 b black body
54.5 pr purple eyes
centromere
67.0 vg vestigial wings
75.5 c curved wings
93.3 hy humpy body
104.5 bw brown eyes
III
0.0 ru roughoid eyes
26.3 h hairy body
44.0 st scarlet eyes
centromere
49.7 ma maroon eyes
62.0 sr striped body
70.7 e ebony body
91.1 ro rough eyes
100.7 ca claret eyes
IV
centromere
0.2 ey eyeless

doubly recombinant. As a result, we have underestimated the percentage recombination between *b* and *vg* by *twice* the frequency of the b^+ cn^+ *vg* and *b cn* vg^+ gametes; that is

$$\frac{2 \times (6 + 9)}{2000} \times 100 = 1.5 \text{ per cent}$$

and the percentage recombination between *b* and *vg* should be 16.25 + 1.5 = 17.75 per cent.

A partial linkage map of the four chromosomes of *D. melanogaster* is shown in figure 5.10.

Genetic Interaction

In the examples described, the pairs of genes segregating have affected widely contrasting characters, shown complete independence in their action and each allelic pair has apparently affected only a single character. But the action of genes in a higher organism fits into a complex integrated network of developmental reactions so that it would be most unusual for a particular cell type, tissue or organ to be determined by the action of just one gene—it would be naïve to suppose that *vg* is the only locus concerned with wing development in *Drosophila*. Furthermore, some genes will have a pleiotropic effect on the phenotype and will affect a number of sometimes apparently unrelated characters—in chordates a mutation which affects the formation of bone may influence the formation of the entire skeletal system and, in turn, this may indirectly affect the development of other organs in the body by, for example, abnormally constricting the space in which an organ must develop.

The formation of pigment in maize illustrates the interaction of several genes in the simplest possible way.

Pigmentation in the Aleurone of Maize

Many genes are involved in the formation of anthocyanin pigments in the aleurone, but there are four basic genes that must be present if any pigment is to form. These are A_1 (located on chromosome 3), A_2 (on chromosome 5), *C* (on chromosome 9) and *R* (on chromosome 10). When these four dominant genes are present the aleurone is normally red, but in the presence of a further

Figure 5.10 A partial linkage map of *Drosophila melanogaster*. The map shows the location of some of the better known mutants of *Drosophila melanogaster*. The map distances are expressed in units of percentage recombination from one end of each chromosome. Note that any pair of markers more than 50 map units apart will assort independently and so appear to be unlinked and that maps with distances of over 50 units can only be constructed from a series of crosses. Using, for example, markers on chromosome II, a three-point cross can establish the order *du*–18–*d*–17–*b* and another cross the relationship *d*–17–*b*–28–*c*. Combining these maps we get *du*–18–*d*–17–*b*–28–*c*; *du* and *c* are said to be 63 map units apart, the sum of the separate map distances between each intervening pair of markers.

dominant gene, *Pr*, (on chromosome 5) the red pigment is converted to purple. The five genes control sequential steps in the biosynthesis of the purple pigment

$$\begin{array}{ccccccccc} \text{W} & \longrightarrow & \text{X} & \longrightarrow & \text{Y} & \longrightarrow & \text{Z} & \longrightarrow & \begin{array}{c}\text{red}\\ \text{pigment}\end{array} & \longrightarrow & \begin{array}{c}\text{purple}\\ \text{pigment}\end{array} \\ & & \uparrow & & \uparrow & & \uparrow & & & & \uparrow \\ & & A_1 & & A_2 & & C & & R & & Pr \end{array}$$

so that if A_1 or A_2, or C or R is not present no pigment is produced, irrespective of whether *Pr* or *pr* is present. A_1 probably specifies an enzyme which converts a colourless compound W into another colourless compound X, A_2 an enzyme which converts compound X into compound Y and so on (the intermediate compounds have not yet been identified).

Consider the cross $A_1A_1\ A_2A_2\ RR\ cc \times A_1A_1\ A_2A_2\ rr\ CC$. Both parent plants will have been grown from kernels with colourless aleurones, but the F_1 hybrid will be uniformly *Rr Cc* (omitting the wild type A_1 and A_2 alleles which are uniformly heterozygous and not segregating) and all the kernels that develop will have coloured aleurones (red or purple, depending on whether *Pr* is present). If the F_1 plants are selfed (i.e. *Rr Cc*⊕) the F_2 progeny phenotypes will be

9	*R C*	coloured aleurone
3	*r C*	colourless aleurone
3	*R c*	
3	*r c*	

The aleurone will only be coloured if both *R* and *C* are present and have interacted to produce pigment; thus there will be a ratio of nine coloured to seven colourless aleurones among the F_2 kernels.

If instead we had crossed *RR pr pr* × *rr Pr Pr* and then selfed the F_1 (*Rr Pr pr*⊕) the F_2 phenotypes would have been

9	*R Pr*	purple aleutone
3	*R pr*	red aleurone
3	*r Pr*	colourless aleurone
1	*r pr*	

and the ratio will be nine purple to three red to four colourless kernels.

So far we have only considered situations where there are two alleles at each locus; there may be more, constituting multiple alleles or a multiple allelic series. For example, at the *C* locus there is a third allele (in addition to *C* and *c*) designated C^I. C^I is a dominant inhibitor and prevents the normal *C* allele from functioning. Thus aleurones which are $C^I\,C^I$, $C^I\,C$ or *cc* will all be colourless, C^IC^I and C^Ic because no *C* allele is present, and $C^I\,C$ because C^I inhibits the action of *C*.

If a $C^I\,C\ R\,r$ is selfed the progeny genotypes and phenotypes can be represented.

9	$C^I\,R$	colourless aleurone
3	$C^I\,r$	colourless aleurone
3	$C\,R$	red aleurone
1	$C\,r$	colourless aleurone (no *R* is present)

then the 9:3:3:1 ratio has been modified to thirteen colourless to three red aleurones.

A good example of multiple alleles occurs at the *R* locus (table 5.4). Note that *R* may also affect the colour of the seedling.

Table 5.4 Multiple alleles at the *R* locus in maize

Allele	Phenotype of aleurone	seedling
R^r	red	red
R^g	red	green
r^r	colourless	red
r^g	colourless	green
R^{st}	stippled red	green
R^{mb}	marbled red	green

In the pericarp the formation of pigment depends on the presence of two dominant genes, A_1 (chromosome 3) which converts a colourless precursor into a brown compound, and *P* (chromosome 1) which converts the brown compound into a purple pigment. Thus a_1 *P* and a_1 *p* pericarps are colourless, *A p* pericarps are brown and A_1 *P* pericarps are purple. It is interesting to note that whereas in the aleurone A_1 A_2 *C* and *R* and *Pr* are all necessary for the formation of purple pigment, in the pericarp only A_1 and *P* are necessary; A_1 is

Table 5.5 Multiple alleles at the *P* locus in maize

Allele	phenotype of Pericarp	Cob
P^{rr}	red	red
P^{rw}	red	white
P^{wr}	white	red
P^{cr}	white with a red cap	red
P^{cw}	white with a red cap	white
P^{or}	orange	red
P^{ww} (or *p*)*	white	white

* p^{ww} and *p* are alternative ways of representing the recessive allele

expressed in both the aleurone and pericarp while the other genes are expressed in only one or the other.

Remember that the pericarp is diploid *maternal* tissue and so on any cob the pericarp of each and every grain has the same genotype. If a *Cc Pp* plant is selfed (A_1, A_2 and R being homozygous) C and c will segregate on the cobs and there will be a 3:1 ratio of red to colourless kernels so that the F_1 can be scored directly on the mother plant. But the segregation of P and p will affect the pericarp and this can only be scored by growing each grain into a mature plant, crossing each plant and then examining the colours of the pericarps.

The P alleles also affect the colour of the axis of the cob, and there is a further series of multiple alleles at this locus (table 5.5), and usually colour in any part of the pericarp or cob is dominant to the absence of colour.

Many other loci also affect the colour of either the aleurone or pericarp.

Continuous Variation

In the examples we have so far considered the genes have interacted to produce several discrete or discontinuous phenotypes and each individual clearly belongs to either one phenotypic class or another—thus each maize grain can be scored as having a purple, a red, or a colourless aleurone. Other characters cannot be classified in this way as there is a continuous range of phenotypes from one extreme to the other. The height of maize plants is one example of a character inherited in this way—clearly we cannot classify a plant as either 'tall' or 'short' because all possible intermediate heights exist in the population.

First, let us consider what happens when just one pair of alleles, A and a, is segregating and when there is no dominance—that is to say, when AA homozygotes are more extreme than Aa heterozygotes. In the cross $Aa \times Aa$ there will be a ratio of 1:2:1 AA:Aa:aa progeny genotypes and phenotypes. Suppose now that two pairs of alleles, A, a and B, b, are segregating and that A and B have similar and additive effects, so that $Aa\ Bb$, $AA\ bb$ and $aa\ BB$ genotypes have the same phenotype; there will now be four phenotypic classes in a 1:3:3:1 ratio—in a like way when six pairs of alleles are segregating there are seven classes in a 1:6:15:20:15:6:1 ratio, so that as the number of gene pairs segregating increases so does the number of phenotypic classes; at the same time the difference between each class decreases and is further diminished by the effects of the environment acting upon the genotypes. As a result, when the number of gene pairs segregating is very large separate classes will not be recognisable and there will be a continuous distribution of phenotypes (figure 5.11).

Many characters, such as height in man, carcase weight in cattle, yield of crop plants, are inherited in this way and are due to the combined effects of very many pairs of genes each contributing a very small but additive effect towards the phenotype. Although the effect of individual genes is much too small to be measured, these genes mutate, segregate and show linkage and recombination in just the same way as the other genes we have examined. Although these

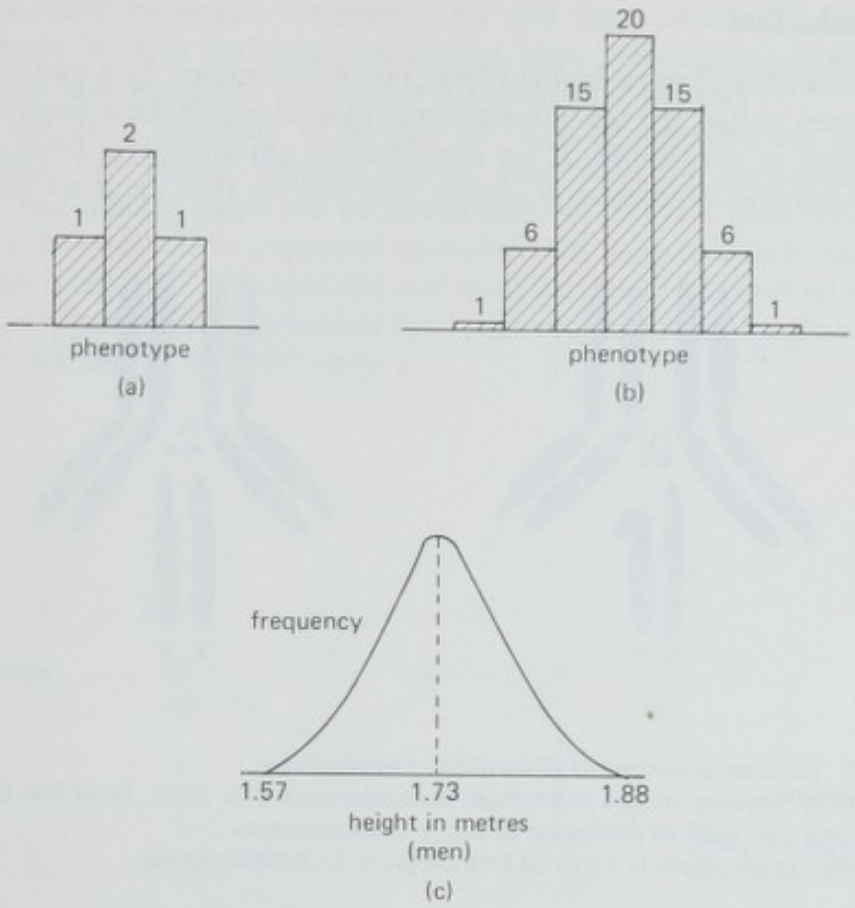


Figure 5.11 Continuous variation.

(a) The segregation of a single pair of alleles will, when there is no dominance, produce three phenotypes in a 1:2:1 ratio.

(b) When there are six pairs of alleles segregating and all affect the same character there are seven phenotypes. As the number of pairs of alleles increases the distinctions between the phenotypic classes diminish until eventually the small remaining differences are smoothed out by the environment, producing a continuous distribution.

(c) Height in man is determined by very many pairs of alleles each with a very small but cumulative effect so that the distribution of height in a population follows a *normal*, or gaussian, curve.

polygenes cannot be studied by Mendelian methods, they do behave according to the neo-Mendelian principles of heredity. These genes are studied in populations by the use of statistical techniques outside the scope of this book.

Sex Linkage

The genes we have considered so far have shown no tendency to segregate with a particular sex but other genes, because they are carried on one of the chromosomes assisting in the determination of sex, do not show this independence; such genes and the characters they determine are said to be *sex linked.*

Sex Determination

In many animals and some higher plants sex is determined by a particular pair of chromosomes, called the sex chromosomes to distinguish them from the non-sex chromosomes or *autosomes*. If we compare the chromosomes from a male and female *Drosophila melanogaster* (figure 5.12) we see that there are three pairs of

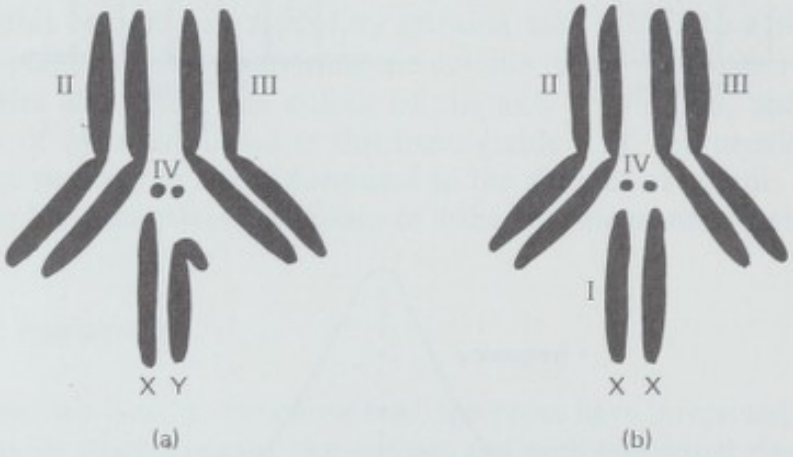


Figure 5.12 The chromosomes of *Drosophila melanogaster*.
(a) Outline drawing of the metaphase chromosomes of a male. Note the three pairs of autosomes and the 'pair' of dissimilar X and Y chromosomes.
(b) In the female there is a pair of homologous X chromosomes.

autosomes common to both sexes, the two large pairs (chromosomes II and III) and the very small pair (chromosome IV). In addition to the two sets of autosomes a female has a pair of homologous X chromosomes (chromosome I) while a male has one X chromosome and a morphologically distinct Y chromosome. These chromosomes are instrumental in determining sex and a zygote with two X chromosomes (XX) will develop into a female and one with an X and an Y chromosome will develop into a male.

This simple description of a *chromosomal* basis for sex determination conceals a variety of *genetic* mechanisms. In man the Y is actively male determining and any individual without a Y is a female (for example, XO, XX, XXX). In *Drosophila*, however, sex depends not on the presence or absence of a Y but on the ratio of the number of X chromosomes to the number of sets of autosomes; individuals with a ratio of 1 or more (for example, diploids with XX or XXX) are female while if the ratio is 0.5 or less they are male (for example, diploid with XO or XY). Clearly in *Drosophila* the genes for determining sex must be carried on both the sex chromosomes and the autosomes.

At meiosis the XX pair in the female and the XY chromosomes in the male segregate normally so that every female gamete contains a single X chromosome (for this reason the female is called the homogametic sex) while the gametes produced by the male are half X and half Y bearing (the male is the heterogametic sex)–thus when the male and female gametes unite at random half the zygotes will be XX (female) and half XY (male). In *Drosophila* and man

(and in all other mammals) the male is XY and the female is XX, but in certain other animals, such as the birds, lepidoptera, some amphibians and some reptiles the situation is reversed and the male is XX and the female XY.

Since the two members of a pair of X chromosomes are homologous, crossing-over can occur between them, recombination frequencies can be measured and linkage maps can be constructed in just the same way as for the autosomes; however, it is important to realise that there is little or no homology between an X and a Y chromosome and most loci on the X have no counterpart on the Y, so that in XY individuals the X-linked genes occur singly instead of in pairs (they are said to be *hemizygous*).

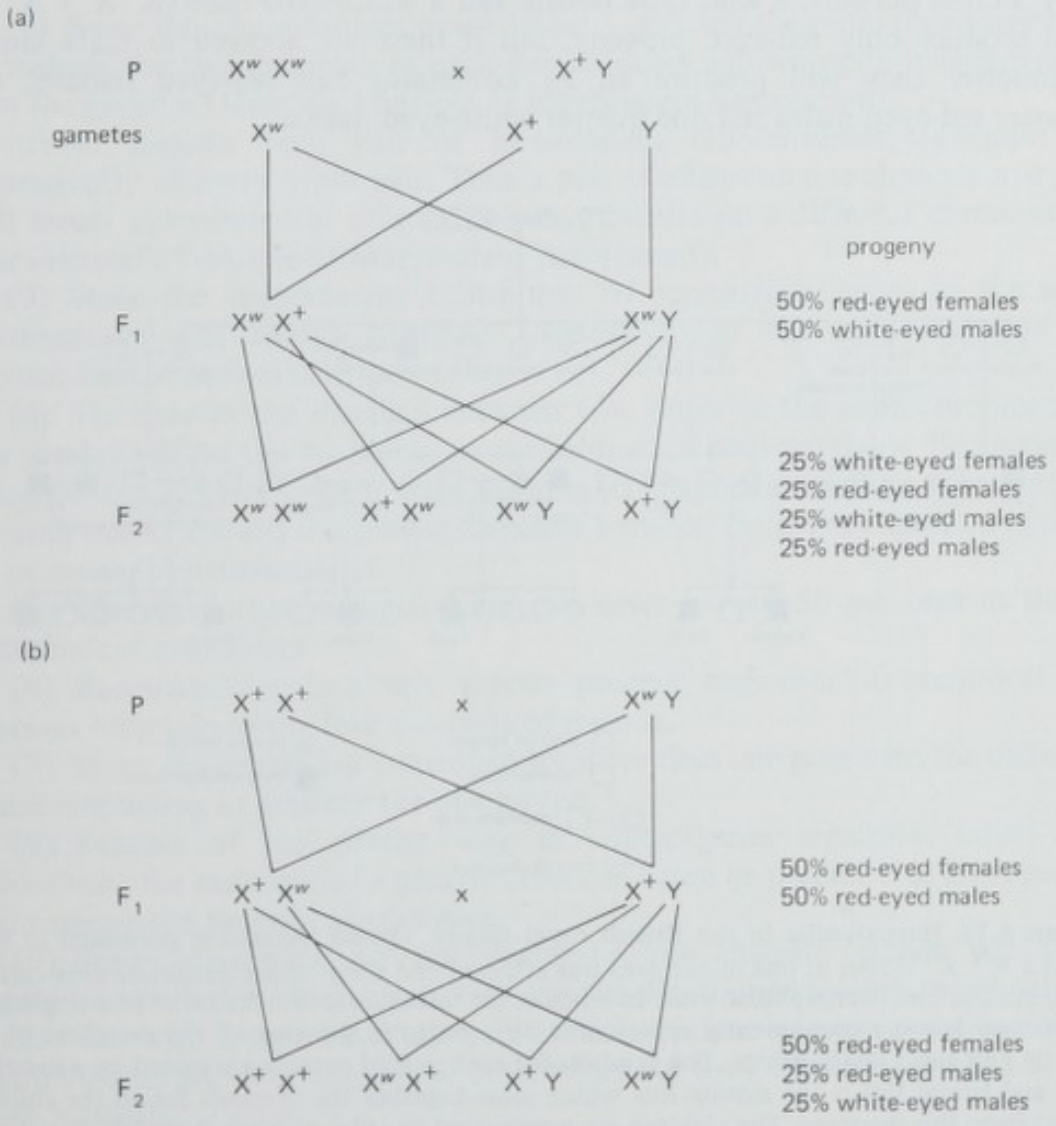


Figure 5.13 The inheritance of a sex-linked recessive character in *Drosophila*. The diagrams show the inheritance of red eye (X^+) versus white eye (X^w).

Sex Linkage

Let us consider the inheritance of the recessive gene *w* located on the X-chromosome of *D. melanogaster;* flies that are homozygous $X^w X^w$ or hemizygous $X^w Y$ have white eyes instead of red eyes. If a white-eyed female ($X^w X^w$—this is a convenient way of indicating that the *w* locus is X-linked) is crossed with a wild type male ($X^+ Y$) *all* the daughters will receive an X^+ chromosome from their father and so will have a wild phenotype (figure 5.13) but all the sons will receive a single X^w chromosome from their mother and a Y from their father and so will have white eyes; the result is an F_1 consisting of all red-eyed daughters and white-eyed sons, showing that the white-eye character is not inherited independently of sex. In the F_2 generation, raised by allowing the F_1 flies to mate among themselves, there will be equal numbers of red-eyed females, red-eyed males, white-eyed females and white-eyed males. In a similar way a cross between a wild type female and a white-eyed male ($X^+ X^+ \times X^w Y$) will produce only red-eyed progeny, but if these are allowed to mate among themselves they will produce an F_2 containing half red-eyed females, one quarter red-eyed males and one quarter white-eyed males.

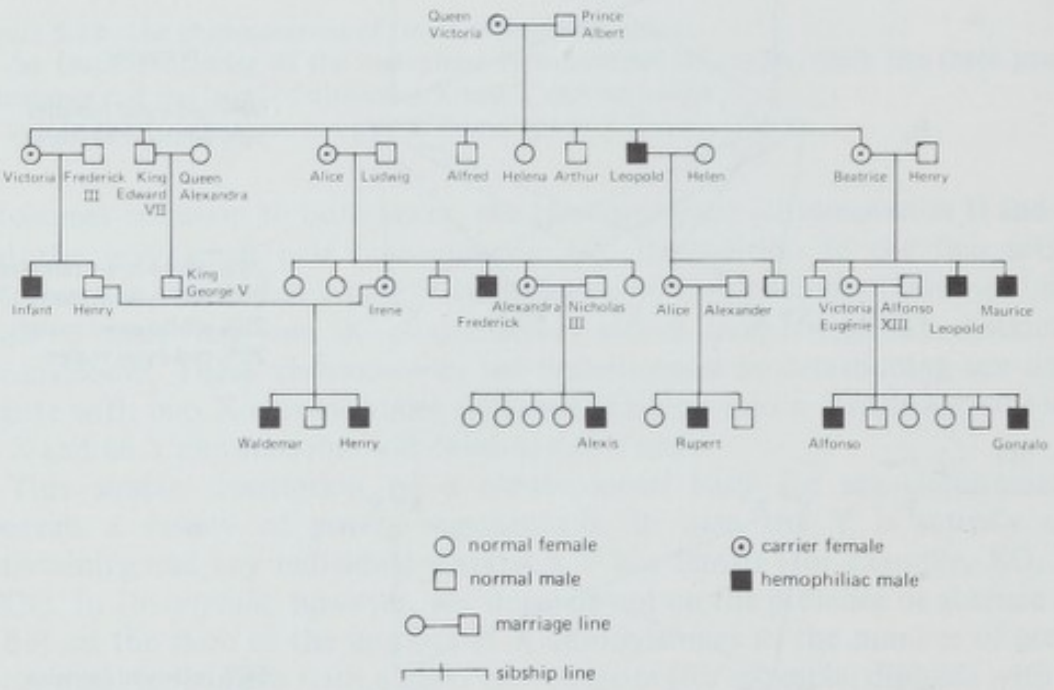


Figure 5.14 Haemophilia in the British royal family. Queen Victoria is presumed to have been a $X^H X^h$ carrier as one of her sons was affected and three of her daughters were carriers and, in turn, had haemophiliac sons. In humans the inheritance of a trait due to a single gene difference is most conveniently represented by a *pedigree* showing all the members of the family and their relationships. The symbols for each pair of parents are joined by a *marriage line* and below this is the *sibship line* which joins together the symbols for all the children (sibs) from this marriage. The children are represented in order of birth from left to right.

Note, firstly, that none of Queen Victoria's ancestors are known to have been affected so that the X^h gene she carried probably arose by mutation, and, secondly, that this deleterious gene is not present in the present royal family.

Sex Linkage in Man

One of the best-known examples of a sex-linked recessive character in man is haemophilia A, bleeders' disease (figure 5.14). The gene responsible, X^h, is quite rare and it is mainly transmitted by heterozygous carrier females (X^h X^H). When a carrier female marries a normal male (X^H Y) one quarter of their children will be haemophiliac sons (X^h Y) and one quarter will be carrier daughters; most of the affected sons will die comparatively young because of the severity of the condition. Affected females are extremely rare as they must have both an affected father and either a carrier or an affected mother; the result is that the character appears to be transmitted through the females of one generation to the males of the next generation.

Summary of Key Points

(1) Since the chromosomes occur in pairs, so must the genes they carry, and at meiosis the two members of each pair of genes will segregate independently into the gametes (Mendel's Principle of Independent Segregation).

(2) At meiosis each pair of homologous chromosomes segregates independently of every other pair. Thus a pair of alleles on one chromosome pair will assort independently of another pair of alleles on a different chromosome pair (Mendel's Principle of Independent Assortment).

(3) Since the chromosome is the unit of segregation, genes on the same chromosome will always segregate together unless they are separated by recombination occurring between them.

(4) The greater the distance between two genes on the same chromosome, the greater will be the frequency of recombination between them. This makes it possible to construct linkage maps showing (i) the positions of the genes relative to each other, and (ii) the genetic distances between the genes expressed in units of percentage recombination.

(5) The percentage recombination can never exceed 50 per cent as this is independent assortment.

(6) Recombination is a very precise process; each event is reciprocal and involves only two of the four products of meiosis.

(7) Many characters are controlled by more than one gene pair, the different genes interacting to produce the phenotype.

(8) Because of the precise way in which genes segregate, assort and recombine, the outcome of a genetic cross can often be predicted as an expected ratio among the progeny phenotypes.

(9) Some characters do not segregate into discrete classes but show continuous variation—such characters may be determined by very many pairs of genes each with a very small but additive effect; these polygenes segregate and recombine in the same way as the other genes but they cannot be studied by the same methods.

(10) In most animals sex is determined by a special pair of sex chromosomes. Genes located on these chromosomes do not assort independently of sex and are said to be sex-linked.

6 Genetic Analysis in *Neurospora* and *Saccharomyces*

Such laboured nothings in so strange a style
Amaze th' unlearn'd and make the learned smile.
Alexander Pope (1688–1744)

Neurospora and *Saccharomyces* are both fungi belonging to the class Ascomycetes and both have been very extensively used in genetic studies, particularly *Neurospora*, the organism used by George Beadle and Edward Tatum in their early experiments leading to the one gene–one enzyme theory (chapter 11). The particular advantages of these fungi are that, on the one hand, they are sexually reproducing and have chromosomes that behave like those in higher animals and plants, and that, on the other hand, like bacteria they have the advantages of a comparatively short generation cycle (the sexual cycle in *Neurospora* can take as little as ten days) and the ability to grow on a simple chemically defined medium containing a carbon source, certain inorganic salts and, in the case of *Neurospora*, the vitamin biotin. From these simple chemicals the fungi can synthesise all the amino acids, vitamins and nucleotides required for growth and reproduction; furthermore, it is a comparatively simple matter to isolate biochemical mutants which are unable to carry out a particular reaction in the synthesis of an amino acid, vitamin or nucleotide and so will only grow when that growth factor is supplied in the medium.

Life Cycles

The life cycle of *Saccharomyces cerevisiae*, bakers' yeast, is quite simple (figure 6.1). The fungus is unicellular and exists in both haploid and diploid phases, both of which can reproduce asexually by mitotic budding. Sexual reproduction also occurs but this requires the union of two haploid cells of opposite *mating types*. Although these mating types are crudely comparable with the two sexes in higher animals and plants there is no morphological differentiation and the differences are purely physiological. Nevertheless, this simple mechanism ensures that a pure strain cannot undergo sexual reproduction and self-fertilisation is prevented. These mating types are usually designated A and a and they are determined by a pair of alleles, *A* and *a*, at a single mating type locus. When two haploid cells of opposite mating type fuse, a diploid cell is produced and this can now undergo meiosis, producing four haploid ascospores held together in a spherical sac-like structure, the ascus; by isolating the ascospores from individual asci it is possible to examine the four products of a

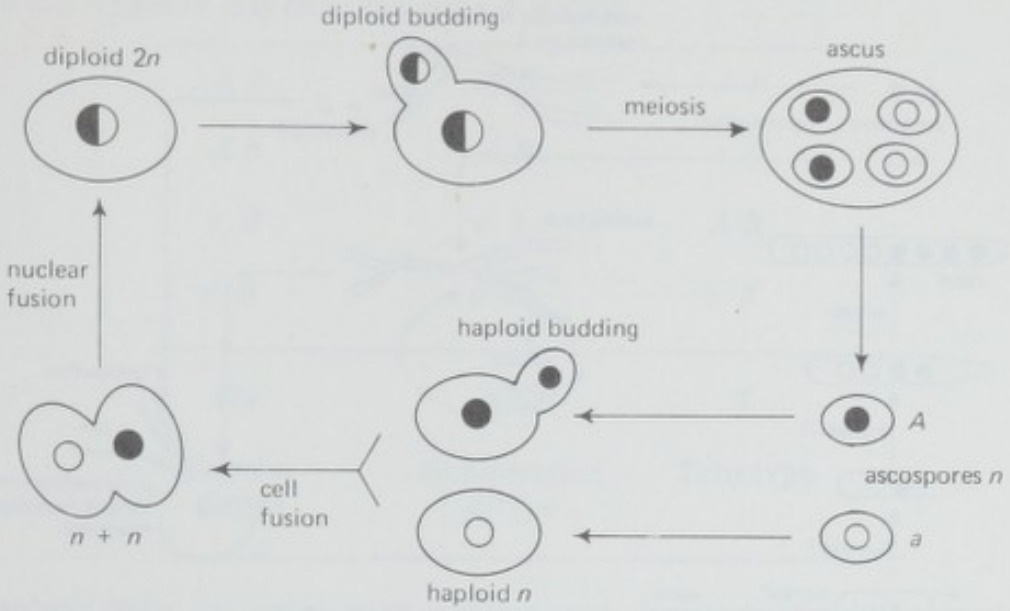


Figure 6.1 The life cycle of *Saccharomyces cerevisiae*.

single meiosis, something that it is not normally possible to do in higher animals and plants. The life cycle is completed by the asci rupturing and releasing the haploid ascospores which now germinate into new yeast cells.

Neurospora crassa is a filamentous fungus and its life cycle is much more complex (figure 6.2). A culture consists of a mass of very fine branched filaments, or hyphae, divided by cross-walls into compartments containing up to 100 haploid nuclei. Reproduction is normally asexual and occurs by the mycelium (that is, the hyphal system) sending up aerial shoots which bear the asexual spores or *conidia*; these may be uninucleate microconidia or multinucleate macroconidia, and they germinate to form new hyphae. Sexual reproduction occurs only when two strains of opposite mating type are brought together. On solid agar both strains produce many female reproductive structures, or protoperithecia, consisting of spherical aggregations of hyphae enclosing a specialised hypha which projects out into the air as the trichogyne. Fertilisation occurs when a microconidium, or sometimes even a piece of mycelium, of the opposite mating type comes into contact with the trichogyne. Fusion occurs and a nucleus from the fertilising cell migrates down the trichogyne and into a special hypha known as the *ascogonium*. The nuclear events that now occur are complicated and in effect the nuclei of both mating types divide and pass in unlike pairs into numerous ascogenous hyphae. In each ascogenous hypha the following events now occur: (1) an *ascus initial* cell is formed containing *one* nucleus of *each* mating type; (2) these two nuclei fuse to form a diploid nucleus in an elongated cell; (3) this diploid nucleus undergoes an immediate meiosis; (4) the four products of meiosis now undergo mitosis to form four pairs of ascospores in an ascus. Meanwhile, other hyphae have formed a thick wall around the ascogenous hyphae forming a flask-shaped *perithecium*.

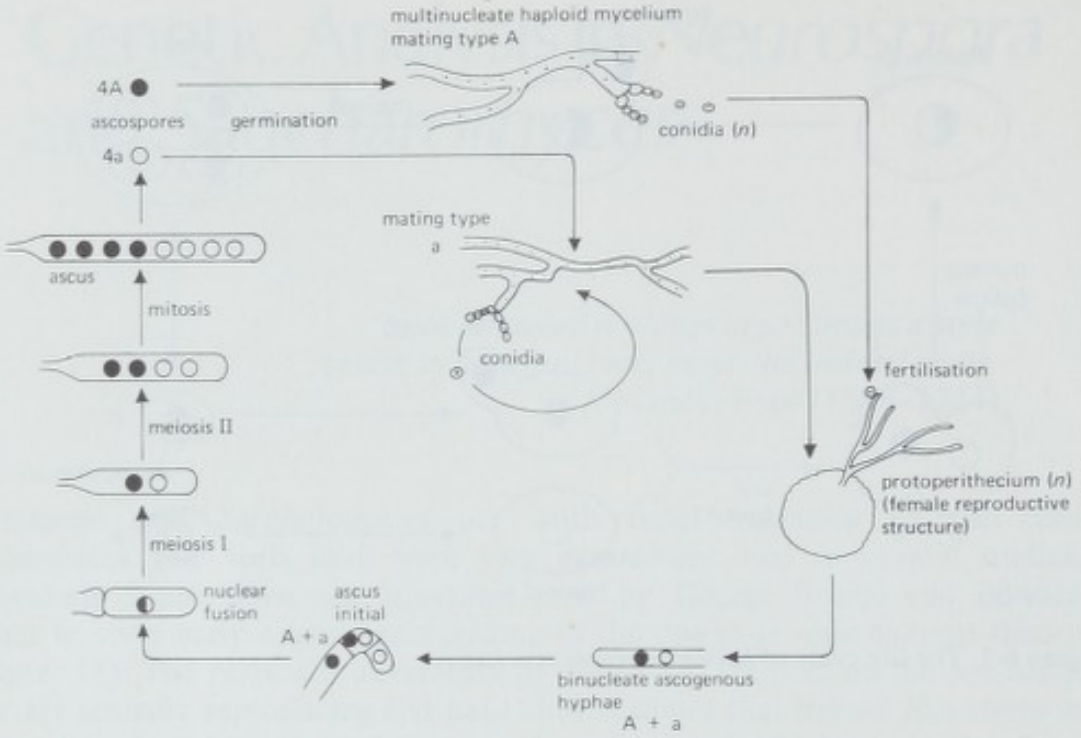


Figure 6.2 The life cycle of *Neurospora crassa.*

It is important to note that each ascus is the product of a single meiosis and that each pair of spores is the product of a mitotic division so that both members of the pair are of the same genotype. Not only have the four products of meiosis remained together as a *tetrad* but they are arranged in a linear order with the ascus. This is an *ordered* tetrad, a very important structure that offers unique opportunities for genetic analysis. The special advantages of tetrad analysis are that (i) linkage relationships can be calculated from the relative frequencies of the different types of tetrad; (ii) it is possible to calculate the linkage between a marker and its centromere–this is straightforward in *Neurospora* but more difficult in *Saccharomyces* with its unordered tetrad; (ii) the precisely reciprocal nature of the ascospores within an ascus shows that meiosis is a reciprocal process; (iv) strand analysis confirms that each cross-over involves only two or the four chromatids, but that multiple cross-over events can involve two, three or all four chromatids of a bivalent.

Tetrad Analysis in Yeast

Yeast, like most other ascomycetes, has an unordered tetrad, but because the four ascospores within an ascus are the four products of a single meiosis there will be 2:2 segregation for each pair of alleles segregating within each ascus. If we cross two strains *Ab* and *aB*, the resultant diploid cells will be doubly heterozygous (*Aa Bb*) and meiosis will produce only three different types of ascus (table 6.1); no other types are possible as no account can be taken of the relative positions of the spores within each ascus.

Table 6.1 Types of asci from the cross *A b* × *a B*

A b	*A B*	*A b*
A b	*A B*	*a B*
a B	*a b*	*A B*
a B	*a b*	*a b*
PD	NPD	T
Parental ditype	Non-parental ditype	Tetratype

The parental ditype asci contain no recombinant spores, the non-parental ditype contain four recombinant spores and the tetratype contain two recombinant and two non-recombinant spores.

In higher organisms the analysis of tetrads is rarely possible and linkage is detected by comparing the frequencies of the parental and recombinant types of gametes. In both *Saccharomyces* and *Neurospora* linkage can also be detected by looking at the relative frequencies of the four types of ascospore after they have been released from the asci and mixed together at random. The method is very simple and rapid as it does not involve the dissection of asci and the isolation of individual ascospores and large numbers of spores can be tested; it is the method usually used in preliminary mapping experiments.

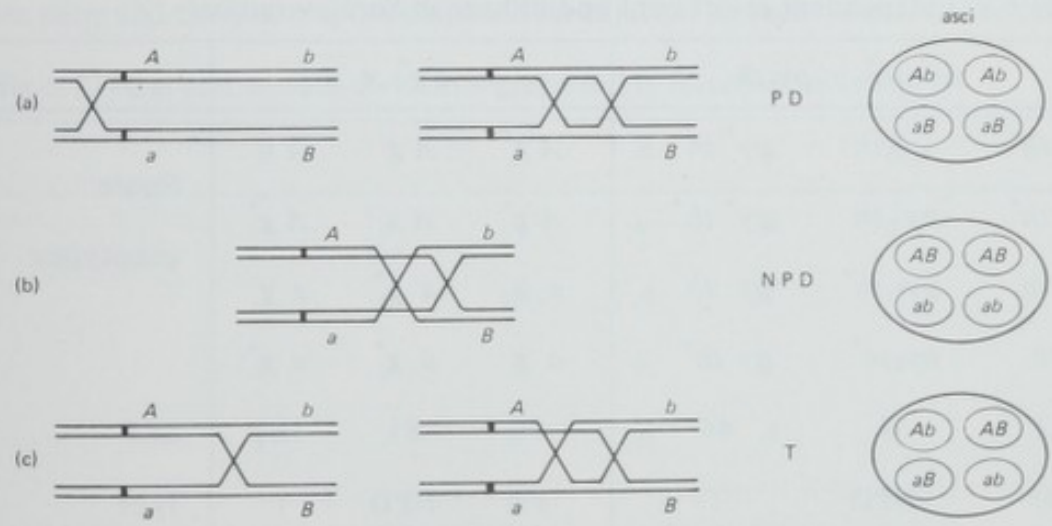


Figure 6.3 The origin of the three types of unordered tetrad. These are the three only possible types of unordered tetrad. Note that a two-strand double cross-over is the equivalent of no cross-over (a) while the three-strand double is the equivalent of a single cross-over (b). PD: parental ditype; NPD: non-parental ditype; T: tetratype.

Let us consider the cross $Ab \times aB$ and assume that the A and B loci are on the same chromosome and linked. A parental ditype (PD) ascus results when there is no crossing over between the A and B loci or when there is a two-strand double cross-over—that is to say, when there are two independent cross-overs involving the *same* pair of chromatids (figure 6.3). A non-parental ditype ascus (NPD) requires all the spores to be recombinant and is the result of a four-strand double cross-over—when there are two independent events each involving a *different* pair of chromatids. A tetratype (T) ascus arises if there is a single cross-over or a three-stand double cross-over, when two independent events involve three of the four chromatids.

Each T ascus contains two and each NPD ascus contains four recombinant chromatids so that the total number of recombinant chromatids is $2T + 4NPD = 2(T + 2NPD)$, where T is the number of tetratype asci, and so on, and the total number of chromatids is $4(T + PD + NPD)$. The percentage recombination between A and B is

$$\frac{\text{number of recombinant chromatids}}{\text{total number of chromatids}} \times 100$$

$$= \frac{2(T + 2NPD)}{4(T + PD + NPD)} \times 100$$

$$= \frac{\frac{1}{2}T + NPD}{T + PD + NPD} \times 100$$

Note that the T asci tell us nothing about linkage as they contain two recombinant and two non-recombinant chromatids just as with independent

Table 6.2 Independent assortment and linkage in *Saccharomyces*

py⁺ th⁺ × py th			*a g⁺ × A g*			
py⁺ th⁺	*py⁺ th*	*py⁺ th*	*A g⁺*	*A g*	*A g*	Spore genotypes
py⁺ th⁺	*py⁺ th*	*py⁺ th⁺*	*A g⁺*	*A g*	*A g⁺*	
py th	*py th⁺*	*py th*	*a g*	*a g⁺*	*a g*	
py th	*py th⁺*	*py th⁺*	*a g*	*a g⁺*	*a g⁺*	
52	4	44	79	81	167	asci
PD	NPD	T	PD	NPD	T	type
PD/NPD = 13.0			PD/NPD = 1			

Data collected by Lindegren for two crosses: *py*: pyridoxine requirement; *th*: thiamine requirement; *g*: galactose non-utilisation; *A*: mating type.

assortment. If there is independent assortment we expect equal numbers of PD and NPD asci (PD/NPD = 1), but if there is linkage there will be more PD than NPD asci (PD/NPD) > 1). the closer the linkage the greater will be the proportion of PD asci, so that if *A* and *B* are very tightly linked there will almost no NPD asci. Table 6.2 shows two sets of data collected by Carl Lindegren. In the cross $a\ g^+ \times A\ g$ the ratio PD/NPD is unity so that *a* and *g* are not linked. On the other hand the $py^+\ th^+ \times py\ th$ cross yielded a large excess of PD asci; *py* and *th* are clearly linked, and applying the formula given above show 26 per cent recombination between them.

Fourteen linkage groups have been identified in *S. cerevisiae.*

Tetrad Analysis in *Neurospora crassa*

The specific feature of *Neurospora* is that its tetrad is ordered. Not only are the four products of meiosis retained together in an ascus but the spores, which represent the four meiotic chromatids, are arranged *linearly* and in the same order as the chromatids were orientated on the metaphase plate at meiosis; thus by genetical methods we can recognise particular chromatids, something that is not possible by cytological examination.

Let us consider what happens if we cross two strains which differ only in respect to mating type. The diploid ascus initials are *Aa* and after meiosis produce two *A* and two *a* products; these divide mitotically to produce four pairs of ascospores. These four pairs of spores can be ordered within the ascus in six different ways (table 6.3); note that for the sake of convenience we write down the genotypes of spore *pairs* rather than of the individual spores. Asci of types 1 and 2 (table 6.3) are known as first division segregation asci because both chromatids carrying *A* move to one pole and the other chromatids carrying *a* move to the other pole at the first meiotic division and in each ascus the two *A* spore pairs and the two *a* spore pairs will lie adjacent to each other. Asci of types

Table 6.3 First and second division segregation in *Neurospora crassa*

	1	2	3	4	5	6
	A	*a*	*A*	*a*	*A*	*a*
	A	*a*	*a*	*A*	*a*	*A*
	a	*A*	*A*	*a*	*a*	*A*
	a	*A*	*a*	*A*	*A*	*a*
asci observed	105	129	9	5	10	16

Lindegren's data (1932) for the segregation of the mating type alleles *A* and *a*. He dissected 274 asci and determined the mating type of each spore pair. The table shows the numbers of each of the six possible types of ascus.

3 to 6 are known as second division segregation asci because *A* and *a* do not separate until the second meiotic division and the identical spore pairs do not all lie adjacent in the ascus.

If the meiotic products were distributed at random the six types of ascus would occur with equal frequencies. The data obtained by Carl Lindegren (table 6.3) clearly shows that this is not so–why? Consider what happens at meiosis. The homologous chromosomes carrying *A* and *a* pair along their length, divide into chromatids and participate in recombination; remember that if the bivalent is to orientate on the metaphase plate and the chromosomes to segregate normally there must be at least one chiasma between the two homologues. Provided that crossing over does *not* occur between the *A* locus and its centromere (figure 6.4a) the two *A* (and the two *a*) alleles will remain attached to the same centromere and will segregate together at the first anaphase and meiosis can only produce an ascus of type 1 or 2. In other words, first division segregation occurs when there has been *no crossing-over between a locus and its centromere.*

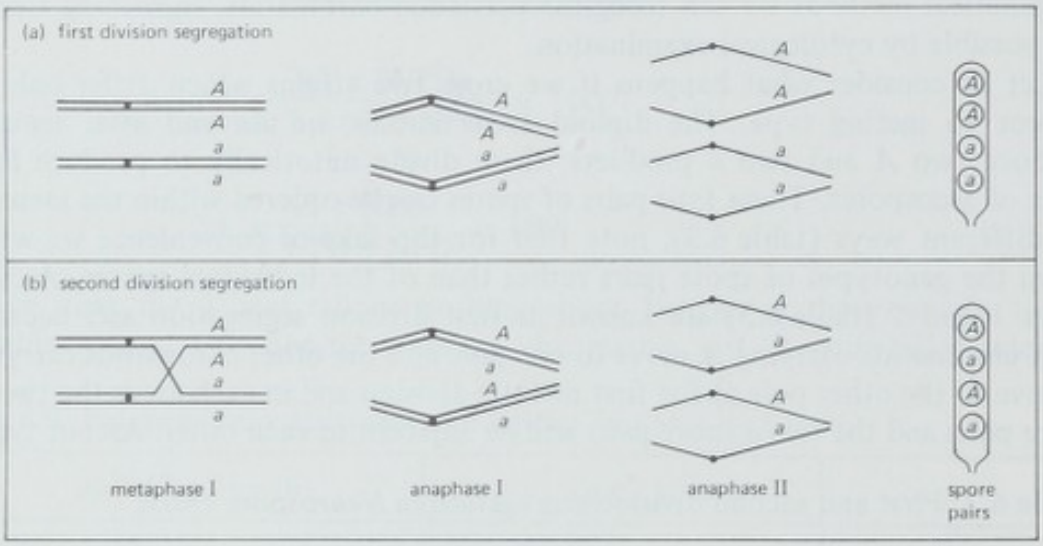


Figure 6.4 First and second division segregation. When there is no cross-over between a locus and its centromere (a) the pairs of sister alleles (*A*,*A* and *a*,*a*) segregate from each other at the first meiotic division; this is first division segregation. When there is a cross-over between a locus and its centromere this segregation does not occur until the second meiotic division (b); this is second division segregation.

Let us now see what happens when there is a cross-over between the *A* locus and its centromere (figure 6.4b). After the first meiotic division each daughter nucleus will receive a chromosome with one *A* and one *a* chromatid. Only at the second meiotic division, when each chromatid (now technically a chromosome) passes into a separate ascospore, will *A* and *a* separate from each other. In the resulting asci the *A* and/or *a* spore pairs will be separated from each other; each second division segregation ascus is the result of a *single cross-over between the locus being tested and its centromere.*

This unusual situation makes it possible to calculate the percentage recombination between a locus and its centromere. The percentage recombination is given by the standard formula

$$\frac{\text{number of recombinant chromatids between } A \text{ and its centromere}}{\text{total number of chromatids}} \times 100$$

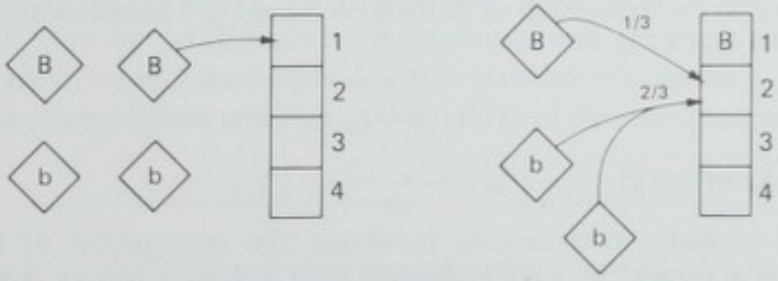
Among the first division segregation asci none of the chromatids is recombinant between *A* and the centromere. In each second division segregation ascus two of the four chromatids (that is, ½) are recombinant, so that among all such asci the number of recombinant chromatids is twice the number of second division segregation asci ($2N_2$). The total number of chromatids is four times the number of asci ($4N_1 + 4N_2$, where N_1 and N_2 are the numbers of first and second division segregation asci) and the percentage recombination is

$$\frac{2N_2}{4(N_1 + N_2)} \times 100$$

which is equal to one half the percentage of second division segregation asci. From Lindegren's data we can calculate the percentage recombination between *A* and its centromere as

$$\frac{1}{2} \times \frac{40}{274} \times 100 = 7.3 \text{ per cent}$$

One point that often causes confusion when mapping markers in relation to their centromeres is that a marker can never show more than 33.3 per cent recombination with its centromere, as any value over this is independent assortment. Consider the segregation of a pair of alleles *B* and *b*, where the *B* locus is so far from its centromere that it is assorting independently. As a result of meiosis we have four chromatids, two carrying *B* and two carrying *b*, to fill the four spaces in the resulting ordered tetrad. If a *B* chromatid segregates into space 1, then 1 *B* and 2 *b* chromatids remain, one of which must fill space 2. Since each chromatid has an equal chance of segregating into space 2, there is a



one in three chance that the *B* chromatid (first division segregation) and a two in three chance that a *b* chromatid (second division segregation) will segregate into space 2. Therefore, if there is independent assortment between a marker and its centromere 66.6 per cent of the asci will show second division segregation, and the percentage recombination value will be half this, or 33.3 per cent.

Table 6.4 Independent assortment in *Neurospora crassa*

	1	2	3	4	5	6	7	Total
	$f^+ A$	$f^+ a$	$f^+ A$	$f^+ A$	$f^+ A$	$f^+ a$	$f^+ A$	
	$f^+ A$	$f^+ a$	$f^+ a$	$f\ A$	$f\ a$	$f\ A$	$f\ a$	
	$f\ a$	$f\ A$	$f\ A$	$f^+ a$	$f^+ A$	$f^+ a$	$f^+ a$	
	$f\ a$	$f\ A$	$f\ a$	$f\ a$	$f\ a$	$f\ A$	$f\ A$	
asci observed	16	20	6	60	3	1	3	109
Asci showing second division segregation for:								
$f^+ - f$				60	3	1	3	67
$A - a$			6		3	1	3	13
Numbers of spore pairs:								
$f^+ A$	32	0	6	60	6	0	3	107
$f\ a$	32	0	6	60	6	0	3	107
$f^+ a$	0	40	6	60	0	2	3	111
$f\ A$	0	40	6	60	0	2	3	111

Lindegren's data on the segregation of fluffy (*f*) *vs* normal (f^+) mycelium and *A vs a* mating types in the cross $f^+ A \times f\ a$.

Independent Assortment

We will now consider two crosses involving the segregation of two pairs of alleles. Table 6.4 shows the data collected by Lindegren from a cross between a wild type strain of mating type *A* and a strain with 'fluffy' mycelium and of mating type *a* ($f^+ A \times f\ a$). Although there are 6 x 6 or 36 different types of asci

when two pairs of alleles are segregating, the number reduces to seven as an ascus of the type

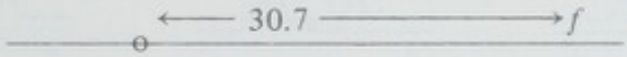
$f^+ A$	can also represent	$f\ a$
$f^+ A$		$f\ a$
$f\ a$		$f^+ A$
$f\ a$		$f^+ A$

while an ascus $f^+ A$ can also represent

$f^+ A$	$f^+ A$	$f\ a$ and	$f\ a$
$f\ a$	$f\ a$	$f^+ A$	$f^+ A$
$f^+ A$	$f\ a$	$f^+ A$	$f\ a$
$f\ a$	$f^+ A$	$f\ a$	$f^+ A$

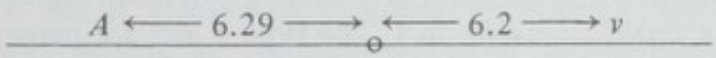
The seven possible types of ascus are shown in table 6.4.

First consider the segregation of f^+ *vs* f. 67 of the 109 asci show second division segregation for f^+ and f, so that there is $67/109 \times ½ \times 100 = 30.7$ per cent recombination between the fluffy locus and its centromere. Similarly there is $13/109 \times ½ \times 100 = 5.96$ per cent recombination between A and its centromere. Second, we must consider whether A and f^+ are on the same or on different chromosomes–is there linkage or independent assortment? This is determined by adding up the frequencies of the four different types of spore pairs (just as in *Drosophila*, except that then we were looking at progeny phenotypes); if f^+ and A are closely linked, we expect a large excess of the parental type spores $f^+ A$ and $f\,a$, but if they are on different chromosomes there should be equivalent numbers of the four types of spore. From table 6.4 we can see that the four types occur at the same frequency so that the A and f loci are unlinked, and the linkage maps can be represented as:

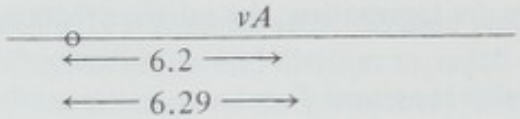


Linkage

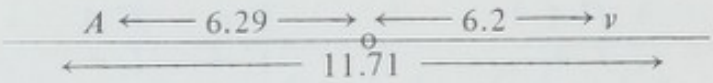
The second cross $A\ v \times a\ v^+$ was carried out by H. Branch Howe (table 6.5). The analysis proceeds in the same way by first calculating the percentage recombinations between A and its centromere (6.29 per cent) and between v and its centromere (6.20 per cent). There are now three possibilities to consider. First, A and v may be on different chromosomes and assorting independently (the large excess of type 1 asci rules out this possibility). Second, A and v may be on the same chromosome and on opposite sides of the centromere.



so that there will be about 12 per cent recombination between them. Third, they may be on the same chromosome arm with only 0.09 per cent recombination between them



If A and v are linked, the percentage recombination is given by the formula *number of recombinant spores/total number of spores* multiplied by 100. There are 544 (11.71 per cent) recombinant spores so that the linkage order must be



Note that 6.29 + 6.2 = 12.49 ≠ 11.71. As we saw in analysing linkage in *Drosophila* there is a discrepancy because in some bivalents there has been more than one recombination event. It is instructive to account for this discrepancy.

Consider the origin of the seven types of ascus. For convenience we call the region between A and the centromere interval I, and the region between the centromere and v interval II. The type 1 asci contain only parental spores and show no recombination in either I or II; all the remaining asci are recombinant in I or in II or in both (figure 6.5). The type 2 asci contain only two types of

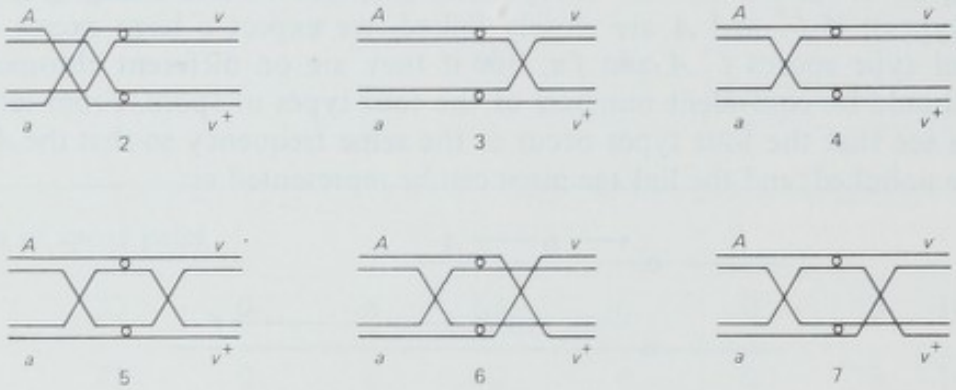


Figure 6.5 Recombination in an ordered tetrad. These diagrams show the origins of the six types of asci containing recombinant spores set out in table 6.5.

spores but they are all recombinant (NPD ascus) although they show first division segregation for both A-a and v^+-v; they have arisen by a four-strand double cross-over in either I or in II. The type 3 asci are tetratype, and show first division segregation for A-a and second division segregation for v^+-v; they are the result of a single cross-over in II. The type 4 asci are similar except that they show second division segregation for A-a and first division segregation for v^+-v; there has been a single cross-over in I. The type 5 asci are parental ditype and show second division segregation for both A-a and v^+-v; they are the result of two-strand double crossing-over in I + II. Asci of type 4 are non-parental ditype and all the spores are recombinant and show second division segregation for both A-a and v^+-v; there has been four-strand double crossing-over in I + II. The type

Table 6.5 Linkage in *Neurospora crassa*

	1	2	3	4	5	6	7
	$A\ v$	$A\ v^+$	$A\ v$	$A\ v^+$	$A\ v$	$A\ v^+$	$A\ v$
	$A\ v$	$A\ v^+$	$A\ v^+$	$a\ v^+$	$a\ v^+$	$a\ v$	$a\ v^+$
	$a\ v^+$	$a\ v$	$a\ v$	$A\ v$	$A\ v$	$A\ v^+$	$A\ v^+$
	$a\ v^+$	$a\ v$	$a\ v^+$	$a\ v$	$a\ v^+$	$a\ v$	$a\ v$
asci observed	888	1	126	128	5	3	10

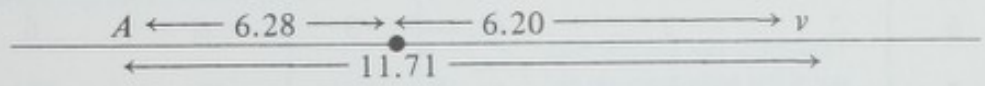
percentage recombination between:

$$A - \text{centromere} = \tfrac{1}{2} \times \frac{(128 + 5 + 3 + 10)}{1161} \times 100 = 6.287 \text{ per cent}$$

$$V - \text{centromere} = \tfrac{1}{2} \times \frac{(126 + 5 + 3 + 10)}{1161} \times 100 = 6.20 \text{ per cent}$$

$$A - v = \frac{(4 + 252 + 256 + 12 + 20)}{4644} \times 100 = 11.71 \text{ per cent}$$

that is



Howe's data (1956) on the segregation of mating type, *A vs a*, and visible slow growth (*v*) *vs* normal (v^+) mycelium in the cross $A\ v \times a\ v^+$.

7 tetratype asci also show second division segregation for both A-a and v^+-v and are the result of three-strand double crossing-over in I + II.

We are interested in only those asci that are the result of more than one cross-over—types 2, 5, 6 and 7. The simplest way to account for the discrepancy is to tabulate the total numbers of chromatids scored as recombinant in I, in II and between A and v^+ (table 6.6). Consider, for example, the five type 5 asci. Each ascus was scored as recombinant in I, recombinant in II but as non-recombinant between A and v^+; thus a total of 5×2, or ten, chromatids were scored as recombinant in I, ten as recombinant in II and 5×0 or none as recombinant between A and v^+. When this is done for each of the four ascus types we see that thirty-six chromatids were detected as being recombinant in I,

thirty-six in II but only thirty-six between A and v^+. Hence, the percentage recombination between A and v^+ has been underestimated by

$$\frac{(72 - 36)}{1161 \times 4} \times 100 = 0.77 \text{ per cent}$$

and

$$\frac{A \longleftarrow 6.28 \longrightarrow \overset{\text{o}}{\longleftarrow} 6.2 \longrightarrow v^+}{\longleftarrow 11.71 + 0.77 \longrightarrow} = \frac{12.48}{12.48}$$

In this example the percentage recombination was underestimated but the bias could have been towards an overestimate had the proportion of type 2 to types 5 and 7 asci been greater.

Table 6.6 How the percentage recombination between $A - v^+$ has been underestimated.

	Numbers of chromatids scored as recombinant between						
	per ascus			No. asci	all asci		
ASCUS TYPE	$A - \bullet$	$\bullet - v^+$	$A - v$		$A - \bullet$	$\bullet - v^+$	$A - v$
2	0	0	4	1	0	0	4
5	2	2	0	5	10	10	0
6	2	2	4	3	6	6	12
7	2	2	2	10	20	20	20
					36 +	36 ≠	36

Extra-Chromosomal Inheritance

In chapter 2 we noted that although most of eucaryotic DNA is localised in the chromosomes there are certain exceptions, notably the DNA of mitochondria and chloroplasts. This DNA forms separate cytoplasmic genetic systems which are replicated and transmitted independently of the chromosomes and so any characters that are determined by genes on this cytoplasmic DNA will show non-Mendelian inheritance.

These cytoplasmic genetic systems appear to be largely independent of the chromosomal DNA, and not only does each organelle (for example, mitochondrion or chloroplast) have its own molecules of duplex DNA, usually in the form of closed circles, but it also has its own DNA and RNA polymerases, ribosomes and transfer RNAs—thus the genetic information on this organellar

DNA is transcribed translated and replicated just as is chromosomal DNA. These DNA molecules vary from 5 μm (animals) up to 30 μm (*Neurospora* or *Saccharomyces*) and it is estimated that each organelle may contain up to twenty molecules of DNA. Only a very limited number of genes could be accommodated on such a small molecule of DNA and there is very good evidence that the structural genes for at least some of the mitochondrial proteins are located on the chromosomes.

One of the best-known examples of extra-chromosomal inheritance is the *petite* character of yeast, first studied by Boris Ephrussi and his colleagues (1950). In cultures of *S. cerevisiae* about 0.5–1 per cent of the cells give rise to colonies that are much smaller than normal; these *petites* lack certain respiratory enzymes and two cytochromes and so cannot grow aerobically. Although the *petite* character is quite stable Ephrussi found that it was immediately lost on crossing and that all four ascospores grew into normal sized colonies; in the same

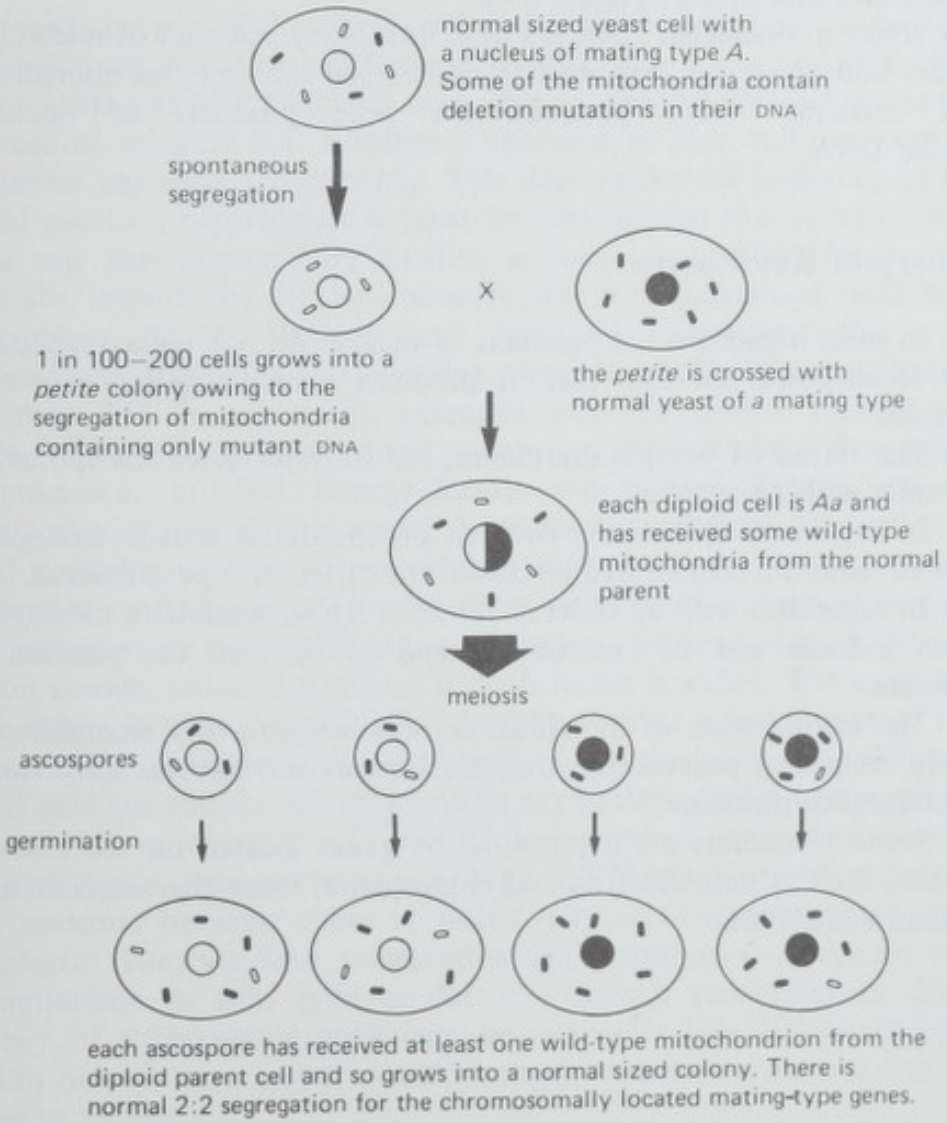


Figure 6.6 Inheritance of the *'petite'* character in *Saccharomyces cerevisiae.*

crosses heterozygous chromosomal markers showed the expected 2:2 segregation. Ephrussi correctly concluded that normal yeast cells contain cytoplasmic particles which enable them to grow aerobically and that in the *petite* strains these particles had all been lost—thus when the *petites* were backcrossed with a normal strain the latter contributed some of these particles to each ascospore so that all the progeny were normal.

More recent work has revealed that the mitochondria of the *petite* cells are unable to incorporate amino acids into protein and it is thought that these mitochondria contain deletion type mutations in their DNA molecules or sometimes contain no DNA at all. Thus the cytoplasmic particles postulated by Ephrussi can be identified as mitochondria containing normal molecules of mitochondrial DNA. Since each cell contains many mitochondria, each in turn containing several molecules of DNA, it is difficult to see how so many daughter cells (10^{-2}) fail to inherit at least one mitochondrion containing normal DNA, but it is a fact that they do (figure 6.6).

The study of organellar DNA is still in its infancy and a lot of research will be necessary before we can even start to understand the complex interactions that almost certainly occur between the gene products of nuclear and organellar DNA.

Summary of Key Points

(1) In most organisms the products of meiosis do not remain associated but in the ascomycetes the four meiotic products remain together as a tetrad of ascospores.

(2) The tetrad of yeast is unordered, but in *Neurospora* the spores are in a linear order within the ascus.

(3) In organisms with either ordered or unordered tetrads, linkage can be calculated from the relative frequencies of the different types of tetrad.

(4) In organisms with an ordered tetrad it is also possible to measure linkage between a locus and its centromere, and so to map the position of the centromere.

(5) The examination of individual tetrads confirms that recombination is a precisely reciprocal process and that each cross-over involves only two of the four products of meiosis.

(6) Some characters are determined by genes located on the DNA of cell organelles, such as mitochondria and chloroplasts; these characters do not show Mendelian segregation.

7 Genetic Transfer in Bacteria: General Transduction and the Analysis of Genetic Fine Structure

Beware that you do not lose the substance by grasping at the shadow.
Aesop (*c.* 550 BC)

During the latter part of the nineteenth century it became clear that bacteria and higher organisms share the attribute of heredity, but it was not until 1943 that Salvador Luria and Max Delbruck were able to establish beyond doubt that the true breeding variants that sometimes appeared in pure cultures were due to spontaneous mutation (chapter 10). This date marks the beginning of modern bacterial genetics; nevertheless it must be remembered that genetic transfer in bacteria was first observed by Griffith as long ago as 1928 (page 14), even though the importance of the discovery was not understood until the early 1940s, when Avery, McCarty and MacLeod, continuing this work on transformation, showed that the active principle in effecting transformation is DNA (page 15). In any event, extensive work on genetic transfer was not possible before 1941 as biochemical mutants, or *auxotrophs* as they are called, were unknown. In 1941 George Beadle and Edward Tatum first isolated biochemical mutants in the mould *Neurospora,* and very soon after Tatum isolated a number of different auxotrophs of *Escherichia coli;* each auxotroph is characterised by its inability to carry out a particular biochemical reaction and so will not grow on minimal medium, a synthetic medium of inorganic salts and a carbon source, unless the missing growth factor is added. The importance of this discovery is that it enabled the development of a wide range of selective techniques. For example, if two different mutant strains are mixed together in minimal medium neither can grow and so any bacteria that do grow must be the result of either mutation of one of the parental strains to prototrophy (prototrophs can grow without added growth factors), or of recombination having occurred between them in such a way as to reconstitute a wild type chromosome. Thus we have a method which enables us to select for very rare back mutations to wild type or for rare genetic recombinants. Since the frequency of these prototrophs may be as low as 1 in 10^9 auxotrophs, the detection of such rare events would be nearly impossible without the use of a selective technique.

Genetic recombination in bacteria (chapter 8) was discovered by Joshua Lederberg and Edward Tatum in 1946 using the coliform bacillus *Escherichia*

coli. They found that when two strains differing by a number of growth factor requirements were grown together in minimal medium prototrophs were repeatedly recovered; these prototrophs could not be explained by mutation since they were never recovered when the strains were grown separately. In one experiment (figure 7.1) they mixed together two multiply marked mutants, one requiring biotin and methionine and resistant to phage T1, the other requiring proline and tryptophan and sensitive to phage T1; samples of the mixture, containing about 2×10^8 bacteria, were pipetted on to and spread over the surface of petri dishes ('plates') containing minimal medium solidified by the addition of agar. After two days' incubation each plate was found to contain 20–30 colonies of bacteria requiring none of the growth factors which served to distinguish the parental strains. In this cross 86 per cent of the prototrophic colonies were resistant to phage T1, while in a similar cross between a mutant requiring biotin and methionine and *sensitive* to T1 and another mutant requiring proline and tryptophan and *resistant* to T1 79 per cent of the prototrophic recombinants were *sensitive* to T1. Thus reciprocally related proportions of progeny were recovered just as in the corresponding coupling and repulsion backcrosses in higher organisms; the alleles for T1 resistance (ton^r) and sensitivity (ton^s) were segregating exactly as if they were linked to the selected markers. These experiments were the first to suggest that the genes of bacteria might be organised into a structure analogous to the chromosome of a higher organism.

The third system of genetic transfer was accidently discovered in 1951 by Norton D. Zinder and Joshua Lederberg while they were looking for recombination in *Salmonella typhimurium*. They inoculated one limb of a Davis

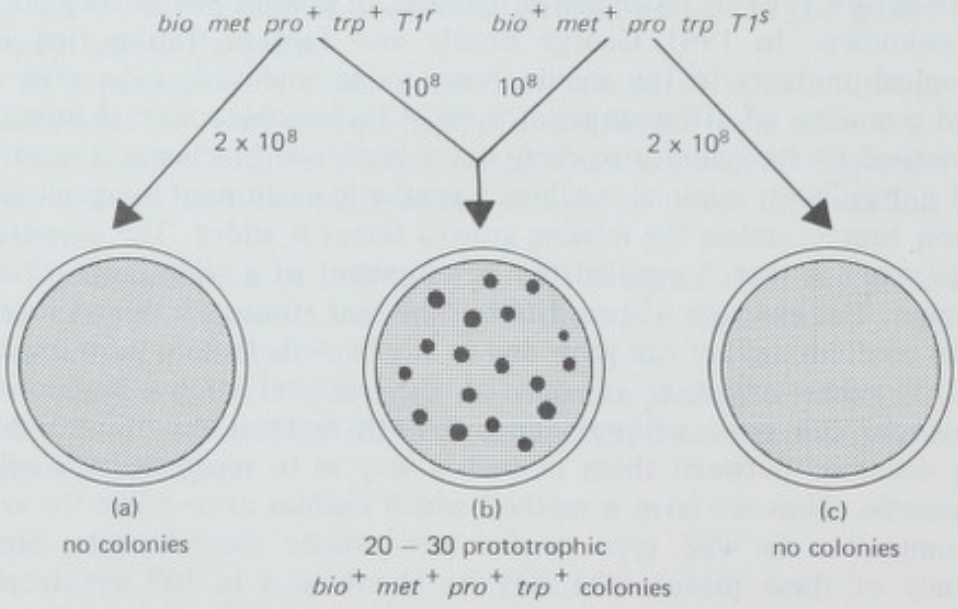


Figure 7.1 Lederberg and Tatum's demonstration of recombination in *Escherichia coli*. When different auxotrophs were plated together on minimal medium many prototrophic colonies were recovered. These were absent from control plates on which only one or other of the parental strains was plated.

U-tube with a histidine-requiring auxotroph and the other limb with a tryptophan-requiring auxotroph that was also lysogenic for phage P22. (In a lysogenic cell a phage chromosome is inserted into the bacterial chromosome and behaves as a stable component of the phage genome. Occasionally, however, the phage chromosome leaves the bacterial chromosome and replicates out of hand; eventually the bacterial cell is lysed, releasing several hundred mature and infective phage particles. Lysogeny is described in chapter 9.) After the liquid in the tube had been sucked back and forth several times between the compartments, Zinder and Lederberg were consistently able to recover prototrophs from among the tryptophan-requiring bacteria (figure 7.2). Since

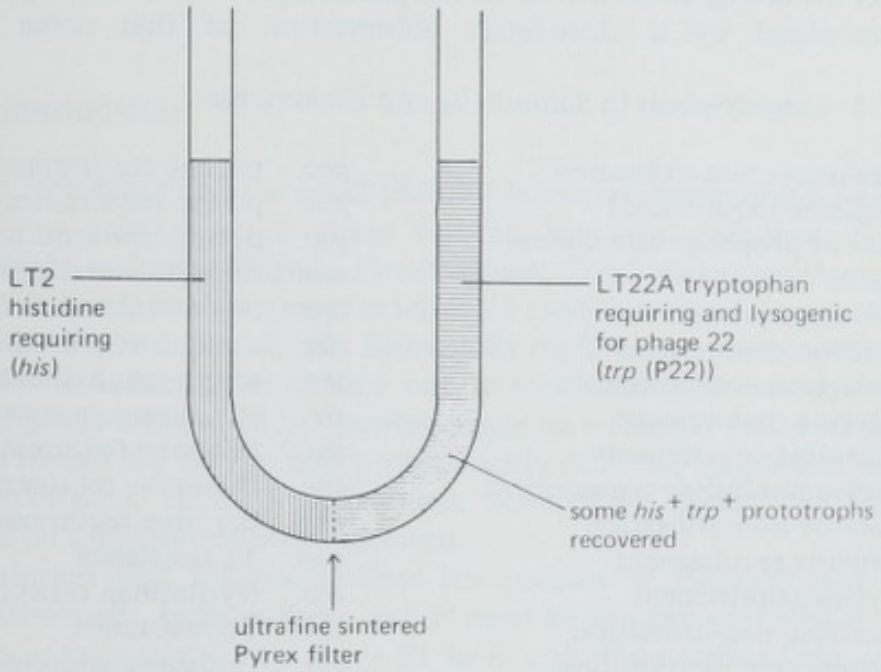


Figure 7.2 The first demonstration of transduction. A histidine-requiring auxotroph of *Salmonella typhimurium* was introduced into one limb of a Davis U-tube, a tryptophan-requiring auxotroph lysogenic for phage P22 into the other limb and the medium sucked back and forth between the compartments. Although an ultrafine filter in the bend of the U-tube prevented any contact between the *his* and *trp* bacteria, some prototrophs were recovered from among the tryptophan auxotrophs.

the limbs of the U-tube were separated by an ultrafine filter, preventing cell to cell contact and the passage of bacteria from one limb to the other, they concluded that the phage P22 particles spontaneously released from the lysogenic tryptophan-requiring mutant passed through the filter, lysed the histidine-requiring bacteria and released from them a filterable agent which could pass back through the filter and endow the tryptophan-requiring bacteria with new genetic properties; in other words, the filterable agent could transfer genetic markers from the histidine-requiring mutant to the tryptophan-requiring mutant (in this experiment the trp^+ marker was transferred). Subsequently, Zinder showed that the filterable agent was phage P22 itself and that the phage particles were capable of carrying fragments of genetic material from the

histidine-requiring *donor* cells to the tryptophan-requiring *recipient* bacteria, where they could then be recombined into the recipient chromosome. They called this process *transduction*, literally 'leading across'.

In this and the next chapter we will examine the processes of transduction and recombination and see how they can be used to analyse the bacterial genome.

The Nomenclature of Bacterial Genetics

The system of nomenclature used in bacterial genetics names each mutant character according to its effect on the phenotype, and the corresponding genes are symbolised by a three-letter abbreviation of that name (table 7.1).

Table 7.1 Gene symbols in *Salmonella* and *Escherichia*

ara	arabinose non-utilisation	*pro*	proline requirement
arg	arginine requirement	*pur*	purine requirement
att	site of prophage attachment	*pyr*	pyrimidine requirement
azi	azide resistance	*ram*	ribosomal ambiguity
bio	biotin requirement	*rec*	recombination deficient
cys	cystine requirement	*rha*	rhamnose non-utilisation
gal	galactose non-utilisation	*ser*	serine requirement
gly	glycine requirement	*str*	streptomycin resistance
his	histidine requirement	*thi*	thiamine requirement
ilv	isoleucine/valine requirement	*thr*	threonine requirement
lac	lactose non-utilisation	*thy*	thymine requirement
leu	leucine requirement	*ton*	T1 resistance
lys	lysine requirement	*trp*	tryptophan resistance
mal	maltose non-utilisation	*tsx*	T6 resistance
man	mannose non-utilisation	*tyr*	tyrosine requirement
met	methionine requirement	*uvr*	uv resistant
mtl	mannitol non-utilisation	*xyl*	xylose non-utilisation
phe	phenylalanine requirement		

The table lists some of the gene symbols used in *Salmonella* and/or *Escherichia* genetics. Specific loci are indicated by including a capital letter in the symbol, for example *leuA*, *leuB*, and so on.

Auxotrophs are named according to their ultimate growth requirement and are numbered in their order of isolation, so that the eighth isolated mutant requiring the amino acid histidine is designated *his-8*. Since each step in a biosynthetic pathway is usually catalysed by the product of a different gene more than one gene is generally involved in the synthesis of a particular end product—the separate genes affecting the same character are distinguished by including a capital letter in the symbol; thus *hisC8* indicates that the *his-8* mutation is located within the *hisC* gene. Sugar fermentation mutants are known by the sugar they are *unable* to ferment so that *ara* mutants will not grow when arabinose is the carbon source but they will grow on glucose; drug and phage resistance mutants are symbolised by a suitable abbreviation with a superscript *r*

or *s* (resistance or sensitivity). All gene symbols are italicised. Similar symbols in roman type, with the first letter capitalised, are used to refer to the phenotype—thus any histidine-requiring auxotroph can be designated His and any prototroph as His^+.

The formula for a transduction is written

hisC8 (×) *trpD10*

with the genotype of the recipient strain on the left and separated from the genotype of the donor strain by the symbol (×), indicating 'transduction'. Note that wherever possible the symbols for the corresponding wild type genes are omitted, but if required they are designated $hisC8^+$, $trpD10^+$ or by just a plus sign alone.

General Transduction

Transduction, the transfer of a small piece of bacterial chromosome from one bacterial cell to another by a phage particle, has been described in many different bacteria, including *Shigella, Pseudomonas, Staphylococcus, Proteus* and *Bacillus subtilis,* but it has been most extensively used to analyse the genetic fine structure of the *Escherichia coli* and *Salmonella typhimurium* chromosomes. In the laboratory transduction is carried out in two stages; first a bacteria-free suspension of phage is prepared by growing phage on a suitable donor strain and, second, these phages are used to infect a genetically different recipient strain and genetic recombinants, called transductants, selected by plating the infected recipient cells on a suitable selective medium.

The description that follows outlines the process of P22-mediated transduction in *Salmonella typhimurium,* and it must be remembered that in other systems, such as transduction by phage P1 in *E. coli,* the details of the process will differ. In *S. typhimurium* transducing phage is prepared by infecting about 10^8 donor bacteria with about 10^6 phage particles; after the phage has replicated and the donor cells have lysed any bacterial debris is removed by centrifugation and the remaining supernatant shaken with chloroform to kill any surviving bacteria. During the lytic growth of the phage in the donor cells the bacterial chromosomes appear to break up into many small fragments, each of which then becomes incorporated into a phage coat instead of a molecule of phage DNA (this is called the 'wrapping-choice' theory). Although a good phage preparation may contain as many as 10^{11} particles per ml, only a small proportion of these will contain bacterial DNA and so be able to effect general transduction. Next, the phage preparation is used to infect a population of recipient cells, usually at multiplicity of infection between 0.1 and 5, and the mixture plated on selective medium. (Multiplicity of infection, M.O.I., is the ratio of phage to bacteria; if the M.O.I. is 5 then, on the average, each bacterium is infected with five phage particles.) Figure 7.3 illustrates transduction when a phage grown on a wild type (leu^+) donor is used to infect a leucine-requiring (*leu*) recipient, and the transduction mixture plated on minimal medium. After 2-3 days' incubation, many leucine-independent colonies are found growing on

the surface of the plates. At this point the importance of always carrying out a suitable *control* must be emphasised, as while most of the colonies are probably transductants some, or all, could be due to back-mutations of the *leu*$^-$ recipient bacteria to *leu*$^+$. An estimate as to how many of these colonies are due to spontaneous mutation is obtained by plating the recipient bacteria alone on the selective medium—any colonies in excess of the number found on these control plates are almost certainly due to transduction.

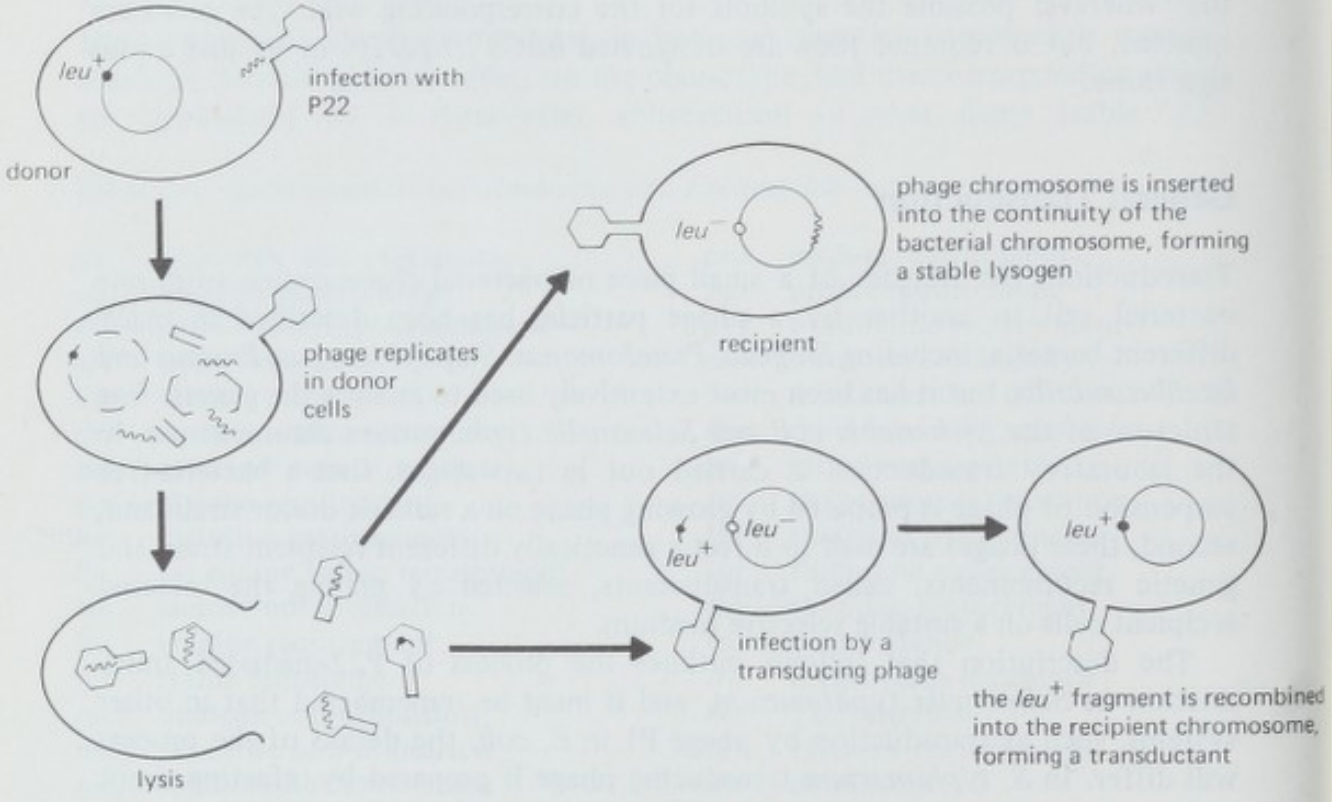


Figure 7.3 General transduction in *Salmonella typhimurium*. When phage P22 is grown on a wild-type (*leu*$^+$) strain the bacterial chromosome fragments and small pieces are incorporated into phage heads instead of phage DNA. One of these transducing particles can now infect an auxotrophic recipient bacterium (*leu*$^-$) and inject into it the corresponding fragment of chromosome from the wild-type donor strain; this can recombine with the recipient chromosome and so transduce the cell to a *leu*$^+$ prototroph. Infection of a recipient cell by a normal P22 particle will either lyse the cell or produce a stable lysogen.

Transduction in the *Salmonella*-P22 system is a rare event—about one transductant for every 10^5 phage particles used—and it can only be detected by using a selective procedure. Any marker can be transferred, hence the name *general* transduction, and it appears that during the growth of the phage on the donor strain small pieces of donor chromosome about twenty to thirty genes long become incorporated into the phage coats instead of phage DNA. Since the *Salmonella* chromosome is long enough to accommodate 2000 to 3000 genes, the fragment must be about 1 per cent of the size of the *Salmonella* chromosome. It is estimated that 0.1 to 0.5 per cent of all the phage particles in

a preparation are transducing particles containing only bacterial DNA, so that for any given gene the frequency of transducing particles will be about $0.3/100 \times 1/100$ or 3×10^{-5}. These transducing particles, because they do not contain any phage DNA, can neither lyse nor establish lysogeny in the cells they infect. A further point to note is that because transduction is such a rare event the simultaneous transduction of two genes on different chromosome fragments is very unlikely indeed, having a probability of about $10^{-5} \times 10^{-5}$ or 10^{-10}; if two genes are consistently transduced together (cotransduced) then they must be carried on the same chromosome fragment and so be very closely linked.

Transduction involves not only the transfer of a chromosome fragment from a donor cell into a recipient cell but also genetic recombination between this fragment and the homologous region of the circular chromosome of the recipient, and in order to generate a stable transductant with an intact and circular chromosome there must be an *even* number of cross-overs; an odd number of cross-overs will produce a linear chromosome containing additional genetic material (figure 7.4) and, presumably, unable to replicate.

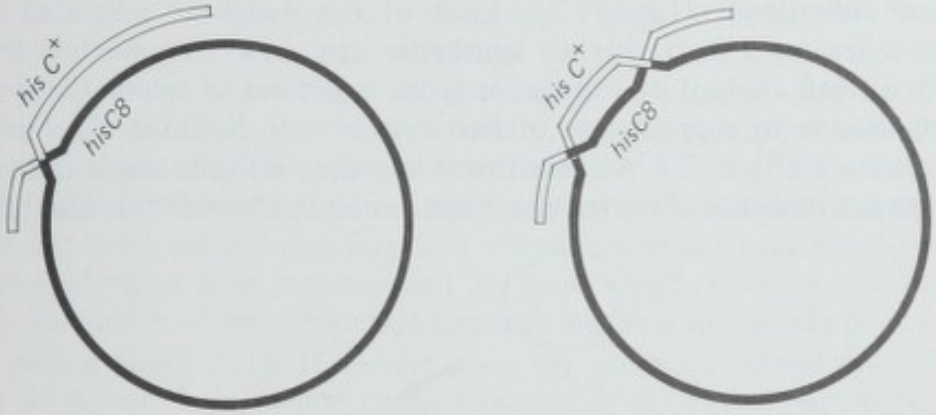


Figure 7.4 The formation of a stable transductant. Genes on the fragment of donor chromosome can be recombined into the homologous region of the recipient chromosome by crossing-over. A single cross-over does not produce an intact recombinant chromosome; this only results when there are two (or some other even number) of cross-overs.

Abortive Transduction

Let us again consider the transduction *leu*$^-$ (×) *leu*$^+$. In a typical experiment each minimal medium plate is inoculated with about 10^8 *leu*$^-$ recipient bacteria and 5×10^7 phage particles and, after incubation, produces 50 to 150 *leu*$^+$ transductants. In addition to these *complete* transductants there are a much larger number, up to about 1300, of very minute colonies called *abortive* transductants; these colonies are usually so small that they can be seen only with the aid of a low-power stereo microscope, and even then it is sometimes necessary to plate on a special medium that restricts the amount of residual growth of the recipient bacteria. About ten abortive transductants are observed for every one complete transductant. Among the 5×10^7 phages used to inoculate each minimal medium plate, a proportion 3×10^{-5} will be trans-

ducing particles carrying the *leu*$^+$ donor genes and so about 1500 *leu*$^-$ recipient bacteria actually receive a fragment of chromosome including a *leu*$^+$ gene. Thus only a comparatively small proportion, usually about 10 per cent, of the *leu*$^+$ genes that are introduced into the recipient cells are actually recombined into the recipient chromosomes to produce complete transductants. The remaining cells give rise to abortive transductants, and although the fragments of donor chromosome are neither able to recombine with the recipient chromosome nor to replicate they are, nevertheless, capable of catalytic activity. Each of these cells is effectively diploid for the *leu* region of the chromosome so that the defect in the recipient chromosome, which is unable to specify one of the enzymes necessary for the synthesis of leucine, can be compensated by the corresponding *leu*$^+$ gene carried on the fragment of donor chromosome (see figure 7.6a); as a result, the abortively infected cell is able to produce all the enzymes required for leucine synthesis and, in turn, to divide. When this cell divides the donor fragment, because it is unable to replicate, is inherited by just *one* of the two daughter cells; this process occurs again and again so that the fragment is inherited along a single cell line—this is called *unilinear inheritance* (figure 7.5). Each of the daughter cells that does *not* receive a fragment is unable to synthesise any new enzyme but it will still contain a small amount of residual enzyme, sufficient to enable the synthesis of enough leucine to support one or two further cell divisions. The products of these divisions (figure 7.5, white arrows), together with the *single* cell containing the fragment of donor chromosome, form a minute abortive transductant.

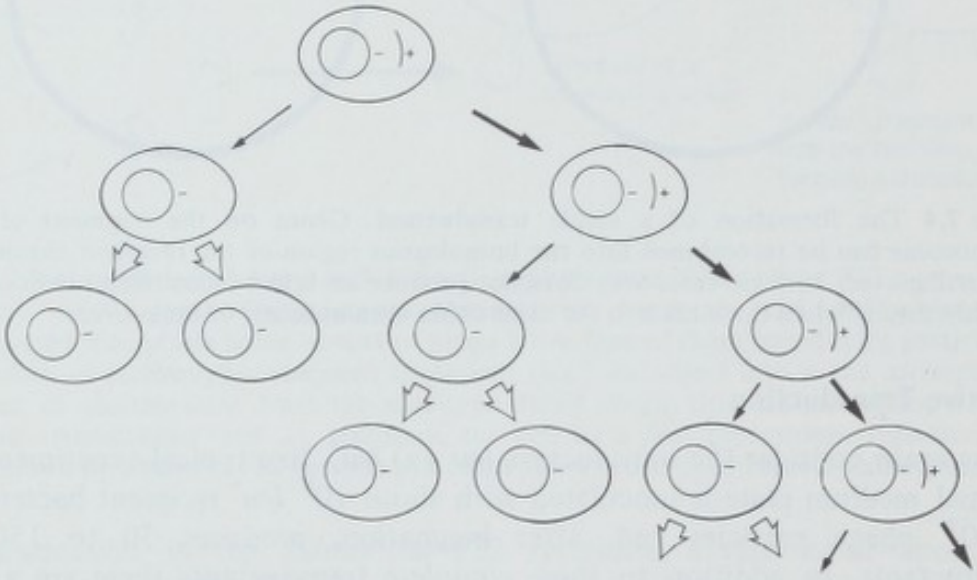


Figure 7.5 Abortive transduction. If, for example, a *leu*$^-$ recipient cell receives a *leu*$^+$ fragment and this fragment does not recombine with the bacterial chromosome, an abortive transductant can result. The infected cell, since it contains a *leu*$^+$ gene, can divide but the fragment does not replicate and is passed into only one of the two daughter cells (large black arrows); the other daughter cell will contain some residual *leu*$^+$ gene product and be able to divide again (white arrows). The process is repeated and the fragment is passed along a single cell line. An abortive transductant consists of the cells indicated by the white arrows, which cannot divide further, and the single cell containing the fragment.

Transduction Analysis in *Salmonella typhimurium*

Until quite recently it was thought that the gene could not be split by recombination and that crossing-over could only occur between genes, but this is not so and most crossing-over, if not all, occurs within the genes themselves. The reason for this misconception was that most of the early mapping experiments were carried out with higher organisms such as *Drosophila*, so that the very rare cross-overs that occur between two extremely closely linked sites within the same gene could only have been detected by the examination of very large numbers of progeny. Although crossing-over between alleles (that is, within a gene) was first demonstrated in *Drosophila* in 1940, it was not until the development of new selective techniques in micro-organisms enabled the rapid screening of very large numbers of progeny that it became generally recognised that the gene consists of a large number of *sites*, which are the units not only of recombination but also of mutation. Each site corresponds to a nucleotide pair in the DNA molecule making up that gene, so that there are as many sites within a gene as there are nucleotide pairs—1000 to 1500 for an 'average' gene.

Genetic analysis has two primary purposes. Firstly, it determines whether independently isolated mutations affecting the same character are due to mutations at different sites within the same gene or whether they are due to mutations within different genes and, secondly, it seeks to arrange a number of different mutant sites into a linear order or linkage map. When the mutant sites being tested are so closely linked on the bacterial chromosome that they are carried on the same transducing fragment, transduction is a very powerful tool for genetic analysis; it is so sensitive and has such a high resolving power that it enables the detection of recombination between adjacent nucleotide pairs within the same codon (page 211). However, when the genes are carried on different fragments of donor chromosome transduction analysis becomes of limited use.

Complementation Analysis

Complementation is a simple *genetic* test used to determine whether two independently isolated mutants are mutant within the same gene (that is, are allelic) or mutant within different genes (that is, non-allelic); it is a standard test that can be used whenever it is possible to put the two mutant chromosomes together in the same cell. In effect, complementation involves the synthesis of an artificial diploid or partial diploid and abortive transduction is just one of the ways that this can be achieved.

If we carry out a transduction between a *leuA* mutant and the wild type (*leuA* (×) *leuA*$^+$) we recover many complete *leuA*$^+$ transductants and a much larger number of abortive transductants—the latter grow because the defect in the mutant *leuA* gene is compensated by the fully functional *leuA*$^+$ gene on the fragment of donor chromosome (figure 7.6a). In just the same way a transduction between two leucine-requiring strains that are mutant in different genes (for example, *leuA* (×) *leuB*) will yield both complete and abortive transductants. In this transduction the abortive transductants arise because the

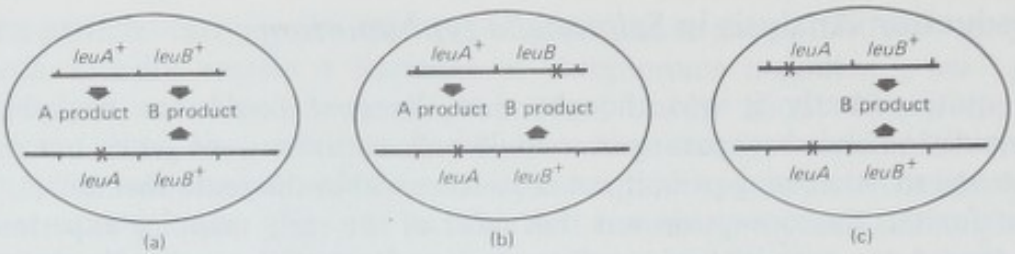


Figure 7.6 Complementation tests.

(a) An abortively infected *leuA* recipient cell that carries a fragment of donor chromosome carrying a *leu*$^+$ gene can form an abortive transductant as the *leu*$^+$ gene on the donor fragment will produce the gene product missing in the recipient.

(b) When a *leuA* recipient carries a fragment of donor chromosome mutant in *leuB* an abortive transductant can still form. Complementation occurs as the gene product missing in the recipient is specified by the corresponding wild-type gene on the donor fragment and vice versa.

(c) When both the recipient chromosome and the donor fragment are mutant in *leuA* neither can produce *leuA*$^+$ gene product and an abortive transductant cannot form.

mutations are in different functional genes and the partially diploid cell is phenotypically wild type (figure 7.6b). Pairs of mutants which make good each other's defects in this way are said to *complement.* However, in a transduction between two different *leuA* mutants recombination can still occur and produce wild type complete transductants but since the abortively infected cells do not contain a functional *leuA*$^+$ gene they are unable to synthesise leucine and no abortive transductants can form (figure 7.6c).

Thus the occurrence of a complementation, here shown by the presence of abortive transductants, indicates that the two mutations involved are in different genes, while the absence of complementation suggests (and no more) that they are in the same gene. The results of some complementation tests using abortive transduction are shown in table 7.2.

Table 7.2 Abortive transduction and complementation

	Donor					
Recipient	Wild type	*leuA121*	*leuB129*	*leuC126*	*leuD128*	*leuCD39*
leuA121	+	–	+	+	+	+
leuB129	+	+	–	+	+	+
leuC126	+	+	+	–	+	–
leuD128	+	+	+	+	–	–
leuCD39	+	+	+	–	–	–

When the mutations in the donor and recipient strains are in different genes, complementation occurs and abortive transductants are recovered (+). When the mutations are in the same gene, no abortive transductants are found (–). Note that *leuCD39* does not give abortive transductants with either *leuC126* or *leuD128*, suggesting that this mutation involves both the *leuC* and *leuD* genes.

In addition to this *intergenic complementation* which occurs between different genes there is another type of complementation which can occur between certain pairs of mutants within the same gene. This intragenic complementation has a quite different explanation and is discussed in chapter 11.

Two-Point Transduction

In higher organisms linkage distances are expressed in units of percentage recombination but in order to calculate these recombination fractions it is necessary to know not only the numbers of each type of recombinant but also the total number of zygotes (chapters 5 and 6). In bacterial systems several reasons combine to make it impossible to calculate map distances in this way. For example, recombination is a very rare event and a selective technique must usually be used to identify *one* specific class of recombinant, all other classes of recombinant being automatically excluded from the analysis; furthermore, it is not possible to estimate the number of non-recombinant zygotes, as these cannot be distinguished from the haploid recipient cells. Other complicating factors are that some bacteria contain more than one nuclear region and that some fragments of donor chromosome may participate in more than one round of recombination.

If the frequency of crossing-over is proportional to distance, an assumption that is made in all mapping experiments, it is theoretically possible to measure the *relative* distances between pairs of markers in units of transduction frequency. The frequency of transduction is usually expressed in terms of either the number of bacterial survivors or of the phage input and with a wild-type donor is of the order of one transductant per 10^5 bacterial survivors or one transductant per 5×10^4 input phages. Unfortunately, however, the frequency of transduction cannot be measured accurately and among duplicate experi-

Table 7.3 Two-point transductions

	Donor							
Recipient	*hisF6*	*hisA33*	*hisH32*	*hisB12*	*hisC15*	*hisC8*	*hisD37*	WT
hisF6	0	825	1403	1125	878	1264	163	4184
hisA33	920	0	290	230	405	566	188	995
hisH32	633	168	0	71	102	238	102	393
hisB12	839	503	156	0	202	311	129	876
hisC15	6184	2488	2602	1676	0	546	132	12244
hisC8	1870	1570	1320	947	377	0	99	4362
hisD37	1198	667	756	535	196	730	0	2810

Phage 22 grown on the donor strains was used to infect 2×10^8 recipient bacteria at a multiplicity of infection of 2.5, and the bacteria plated on minimal medium. The transductants were scored after 48 h at 37° C (P. E. Hartman, 1956).

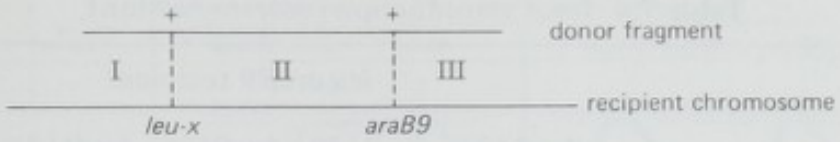
ments it may vary considerably because of differences in the growth phase of the recipient bacteria, the exact composition of the medium, the density of plating and so on. Some data obtained by P. E. Hartman in transductions between different pairs of histidine auxotrophs of *Salmonella typhimurium* are shown in table 7.3. The seven auxotrophs are mutant within six different genes, all closely linked and carried on the same fragment during transduction, and in the table the mutant sites are set out in the same order as they occur on the linkage map. If reliable measurements were possible the frequency of transduction would be lowest between the pairs of sites which are closest together, increasing as the distance between the sites (and so the size of the interval in which recombination must occur) increases. While the data reveal a general trend towards this ideal, the discrepancies are too considerable to allow the construction of a linkage map. For example, note that *hisD37* is consistently ineffective as a donor strain, and that whereas *hisH32* is a very poor recipient *hisC15* is a very effective recipient; clearly genetic as well as environmental factors are influencing the frequency of transduction.

More reliable are methods of analysis that do not involve the estimation of the frequencies of transduction but which *compare* the numbers of different classes of transductant—there is no reason to suppose that variations in the overall frequency of transduction will influence the proportions of the different classes of recombinant. These methods can only be used if there is a second genetic marker linked by transduction to the gene or group of genes being studied; unfortunately suitable closely linked markers are not always available. These methods will be illustrated by P22 mediated transduction of the leucine group of genes in *S. typhimurium*. Four adjacent genes, *leuA*, *leuB*, *leuC* and *leuD*, specify the enzymes involved in the biosynthesis of leucine and are linked by transduction to a sugar fermentation marker, *araB*. Leu$^-$ mutants will only grow if leucine is present in the medium while Ara$^-$ mutants are unable to ferment arabinose. The mutant *leuA121* lacks a synthetase enzyme, *leuB129* a dehydrogenase, *leuC126* and *leuD128* different components of an isomerase while *leuCD39* lacks both the *leuC* and *leuD* gene products. The mutants are all available as double mutants with *araB9* and we require to construct a linkage map showing the order of these mutant sites relative to *araB9*.

The Joint Transduction Method

The first method measures the frequency of joint transduction or cotransduction of each of the *leu* sites with the *araB9* marker. This is achieved by infecting each Leu$^-$ Ara$^-$ double mutant with phage grown on a wild-type donor and selecting leucine-independent transductants by plating on minimal medium. Some of the Leu$^+$ transductants will be Ara$^+$ (the joint transductants), others will be Ara$^-$ and since the selective medium contains glucose both types of transductant will grow equally well. Each Leu$^+$ transductant is then individually tested on indicator medium to ascertain whether it can (Ara$^+$) or cannot (Ara$^-$) ferment arabinose.

If x is a mutant site within the leucine gene cluster, then the transduction *leu-x araB9* (×) + + can be represented



In order to generate a *leu*$^+$ transductant there *must* be one cross-over in interval I (this is called an obligatory cross-over), but the second cross-over can be in either interval III or interval II, generating *leu*$^+$ *ara*$^+$ and *leu*$^+$ *araB9* transductants respectively (figure 7.7). The closer the *leu-x* site to *araB9* the smaller will be

Figure 7.7 Joint transduction. In the transduction *leuA121 araB9* (x) + + *leu*$^+$ transductants are selected by plating on minimal medium. There is an obligatory cross-over in region I, but the second cross-over can be either in II or III, generating *leu*$^+$ *araB9* and *leu*$^+$ *araB*$^+$ transductants respectively.

region II and the fewer the number of cross-overs that will occur within it, and so the higher will be the proportion of *leu*$^+$ *ara*$^+$ transductants due to crossing-over in regions I and III; thus the nearer the *leu* and *ara* sites the higher will be the frequency of joint transductants. In any transduction the size of interval II relative to the size of interval III is given by the percentage

$$\frac{\text{frequency of crossing-over in region II}}{\text{frequency of crossing-over in regions II + III}} \times 100$$

that is, by the percentage of *leu*$^+$ *araB9* transductants.

The results of a series of transductions in which each of the five *leu araB9* strains was used as a recipient with a wild-type donor are shown in table 7.4. Using the arguments just given the *leuA121, leuB129, leuC126* and *leuD128* mutant sites can be arranged on a linkage map in the order of increasing frequency of joint transduction with *araB9*. Observe that once again *leuCD39* has behaved somewhat differently from the other mutants. In previous experiments we saw that *leuCD39* neither recombines with nor produces abortive transductants with either *leuC126* or *leuD128*; in these experiments it shows a much higher frequency of joint transduction with *araB9* than does any other *leu* mutant. The most likely explanation is that *leuCD39* is a *multisite* mutant that arose by the loss or deletion of all the *leuD* gene, all or most of *leuC* and possibly some unknown genes between *leuD* and *araB*. We can represent the linkage map as

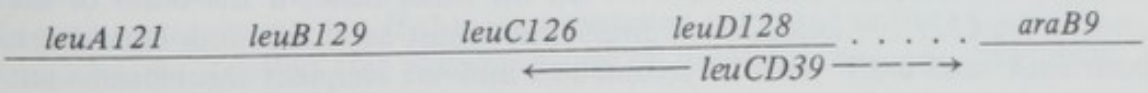


Table 7.4 Joint transduction (cotransduction)

	leu araB9 recipient				
	leuA121	*leuB129*	*leuC126*	*leuD128*	*leuCD39*
leu$^+$ transductants tested	2890	3024	3205	2951	2863
leu$^+$ *araB9* transductants	1607	1565	1615	1315	980
leu$^+$ *ara*$^+$ transductants	1283	1459	1590	1636	1883
% *leu*$^+$ *ara*$^+$ transductants	44.4	48.3	49.6	55.4	65.8

Five different leucine-requiring and arabinose non-fermenting mutants of *Salmonella typhimurium* were infected with phage P22 grown on a wild-type donor, and leucine-independent transductants selected on minimal medium. A proportion of the *leu*$^+$ transductants also inherit the donor *ara*$^+$ marker (joint transductants).

The Three-Point Transduction Method

A more reliable method for establishing linkage order is the three-point test cross. This is essentially similar to the three-point test cross in higher organisms and it seeks to order the markers so that the data can be accounted for by the minimum number of cross-overs. We will discuss one particular set of experiments which enables us to distinguish between the two possible gene orders

(1) *leuC126* . . . *leuA121* . . . *araB9*
and (2) *leuA121* . . . *leuC126* . . . *araB9*

To do this we must have not only the two double mutants (*leuC126 araB9* and *leuA121 araB9*) but also the two single mutants without the *araB9* marker (*leuC126* and *leuA121*); the single mutants are used as donors with the double mutants as recipients, leucine-independent transductants selected by plating on minimal medium and each *leu*$^+$ transductant tested to see whether it is *ara*$^+$ or *araB9*. In these transductions the following proportions of *leu*$^+$ *ara*$^+$ transductants were observed

leuA121 araB9 (x) *leuC126*	6 per cent
leuC126 araB9 (x) *leuA121*	52 per cent

Consider the first transduction *leuA121 araB9* (x) *leuC126*. If the order of the genes is *leuC126* . . . *leuA121* . . . *araB9* only two cross-overs are necessary to account for the *leu*$^+$ *ara*$^+$ transuctants (figure 7.8) and the proportion of these transductants should be about 45 per cent, the same as observed in the transduction *leuA121 araB9* (x) + + . On the other hand, if the order of the genes is *leuA121* . . . *leuC126* . . . *araB9* there must be at least four cross-overs; both *leuA*$^+$ and *araB*$^+$ must be recombined into the recipient chromosome but

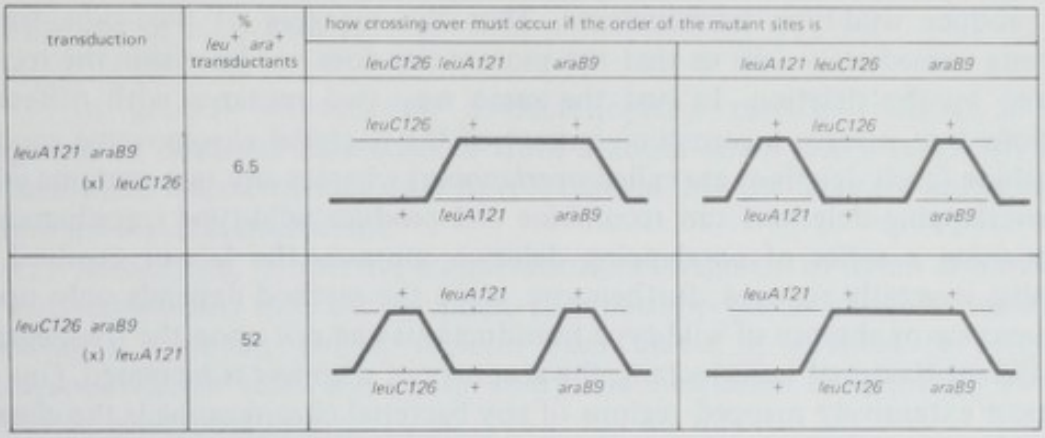


Figure 7.8 Establishing linkage order by three-point transductions. The diagrams show where crossing-over must occur to generate *leu*$^+$ *araB*$^+$ transductants for each of the possible linkage orders.

not leuC126 between them. Since quadruple crossing-over will be rare compared to double crossing-over only a few joint transductants will be expected. As only 6 per cent joint transductants were observed we conclude that the correct order is *leuA121 . . . leuC126 . . . araB9.*

This is confirmed by the result of the transduction *leuC126 araB9* (x) *leuA121.* With the gene order *leuC126 . . . leuA121 . . . araB9* quadruple crossing-over is necessary to account for the *leu*$^+$ *ara*$^+$ joint transductants while with the order *leuA121 . . . leuC126 . . . araB9* only one pair of cross-overs is required. The observed frequency of 52 per cent is too high to be explained by quadruple crossing-over and is almost exactly the frequency of joint transduction observed in *leuC126 araB9* (x) + + (table 7.4). We again infer that the gene order must be *leuA121 . . . leuC126 . . . araB9.*

Two points we must note are, firstly, that the same order would have been obtained if we had selected *ara*$^+$ transductants and characterised these for their ability to grow on minimal medium and, secondly, by combining the results of a series of experiments we can build up a more complete linkage map of the leucine gene cluster.

Deletion Mapping

By far the most accurate method for establishing where the site of a mutation is located is by using multisite mutants. By definition these mutants behave as if they are missing a part of their chromosome and so are often called deletion mutants. If a deletion mutant (for example *leuCD39*) is crossed with a point mutant (a single-site mutant) the outcome will depend on the position of the point mutation in relation to the segment missing in the deletion mutant. If the point mutation is located in the segment of chromosome corresponding to the missing region in the deletion mutant (for example *leuD128*) then recombination obviously cannot restore a wild-type chromosome and generate wild-type transductants, but if the point mutation is located outside the region covered by the deletion (for example *leuB129*) recombination can take place

and produce wild-type transductants. Thus the recovery of wild-type transductants immediately tells us that the mutant site does *not* lie within the region covered by the deletion. In just the same way two mutants with different deletions that involve a common segment of the bacterial chromosome cannot recombine (such deletions are called *overlapping*) whereas any two mutants with non-overlapping deletions can recombine and produce wild-type transductants.

By using a series of overlapping deletion mutants the labour involved in mapping is greatly reduced; furthermore, since the method depends only upon the presence or absence of wild-type transductants and not upon the frequencies of different classes of transductant, the accuracy of mapping is increased. One of the most extensively mapped regions of any bacterial chromosome is the cluster of genes controlling the biosynthesis of histidine in *S. typhimurium* (the functions of these ten genes is described in chapter 14). Many deletion mutants occur in this region and can be used in mapping experiments; a few of these are shown in figure 7.9. To illustrate the principle of deletion mapping let us assume that we have just isolated a new histidine-requiring mutant and wish to establish its map position. We find that in transductions it gives wild-type transductants with all the deletion mutants shown in figure 7.9 *except* numbers 22, 2226,

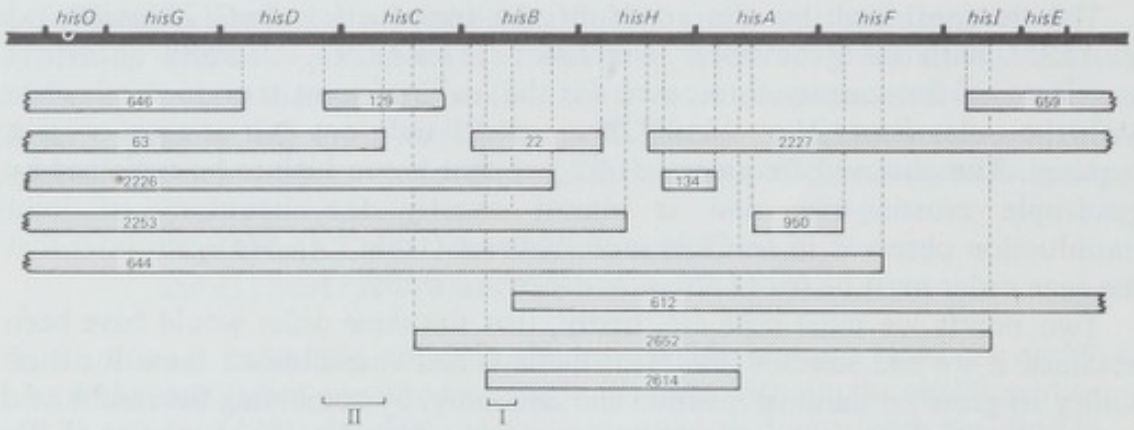


Figure 7.9 Deletions in the histidine gene cluster of *Salmonella typhimurium*. The *his* region consists of ten genes each with a different function in the biosynthesis of histidine. Many deletions are known in this region and some are shown on this map. The heavy horizontal line represents the bacterial chromosome and the short vertical bars separate the ten *his* genes. The regions deleted in fourteen deletion mutants are shown by the grey bars; the vertical zigzag lines indicate that the deletions extend into the adjacent regions of the bacterial chromosome.

2253, 644, 2652 and 2614. Clearly the mutant site must lie in a segment that is common to all these mutants but *not* to any other mutant; the only such segment is in the middle of *hisB* (region I in figure 7.9). Similarly, a mutant that does not recombine with deletions 129, 63, 226, 2253 and 644 but does recombine with all the other deletion mutants must lie near the junction of the *hisD* and *hisC* genes (region II). The exact position of the mutant sites within these very small segments can now be determined by crossing with other point mutations located in these regions.

Summary of Key Points

(1) In general transduction a bacteriophage particle transfers a small fragment of bacterial chromosome from a donor strain into a recipient. These chromosome fragments may then be recombined into the recipient chromosome, forming a stable transductant.

(2) Sometimes, in abortive transduction, the fragment of donor chromosome is not recombined into the recipient chromosome but is inherited unilinearly creating a transient partial diploid. This makes it possible to carry out complementation tests and so to study the functional relationships between different gene mutations.

(3) Each fragment of donor chromosome carries 20–30 bacterial genes and only genes on the same fragment can be transduced together—for this reason transduction analysis is unsuited for investigating the gross genetic structure of the bacterial chromosome.

(4) Transduction analysis not only permits the mapping of the genes carried on each fragment but it also resolves the order of different mutant sites within a gene.

(5) These analyses clearly show that both mutation and recombination occur *within* a gene; although the gene is a unit of function it is not the unit of either mutation or of recombination.

(6) It is not possible to express the distances between genes in units of percentage recombination, but the *relative* distances between closely linked pairs of markers can be expressed in terms of the relative frequencies of crossing-over in each interval.

8 Conjugation and Recombination in *Escherichia coli*

It is to be noted that when any part of this paper appears dull, there is a design in it.

Sir Richard Steele (1672-1729)

Using data from experiments similar to those described on page 106 Joshua Lederberg was, by 1947, able to construct a simple linkage map of the *E. coli* chromosome (figure 8.1a). He made the same assumptions that are made in analysing any linkage data, that the closer are two genes on a chromosome, the more frequently they are transferred together, and he expressed genetic distances in terms of the relative frequency of recombination between each pair of markers. However, two difficulties became apparent as the number of characters studied increased. Firstly, as the intervals between the markers became smaller so the recombination frequencies decreased and it became impossible to plot the positions of the genes with any degree of accuracy. Secondly, although the linkage relationships within each group of markers was clear, it was often impossible to establish the position of these groups relative to each other. For example, Lederberg found that the markers *str, xyl, thi* and *bio* were all linked to *met* (these gene symbols are explained in table 7.1) and yet somehow they appeared to be unlinked to each other; to overcome this anomaly Lederberg proposed that the *E. coli* chromosome was a branched structure (figure 8.1b). In 1953, the classic work of William Hayes clearly established the existence of at least two sexually differentiated types of *E. coli*—males or donor cells and female or recipient cells, which could conjugate in unlike pairs—but the anomaly of the branched chromosome was not resolved until after Elie Wollman, François Jacob and William Hayes established the nature and mechanics of the mating process in 1956 (page 126 *et seq.*).

The male or donor strains are distinguished from the female or recipient strains by possessing an additional genetic unit, the F factor, sex factor or fertility factor. (Contrary to popular usage, the terms donor and recipient are to be preferred to avoid the implication of morphological differentiation such as distinguishes the males and females in higher organisms.) Whereas in transduction genetic material is transferred from one bacterial cell to another by a phage vector, in conjugation the F factor promotes direct contact between a donor and a recipient cell, the formation of a cytoplasmic bridge or conjugation

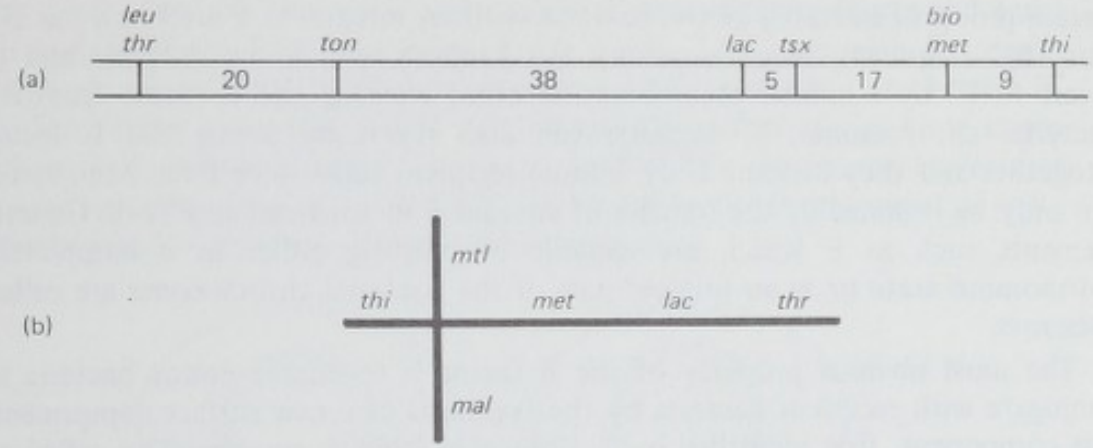


Figure 8.1 The first linkage maps of *E. coli*. The linkage map of the *E. coli* chromosome constructed by Lederberg in 1947 (a) and (b) the branched structure he proposed in 1951 to account for the anomalous linkage data he observed.

The map distances are expressed as the relative frequencies of recombination between the pairs of markers and not in units of percentage recombination.

tube between them and the oriented transfer of genetic material from the donor into the recipient. As we shall see, there are two types of donor strain, F^+ strains which can conjugate but can only transfer the F factor itself, and Hfr strains which are able both to conjugate and to transfer their chromosome into recipient cells.

The F Factor

The F factor is a small molecule of DNA about 6×10^4 nucleotides long (about 2 per cent of the length of the *E. coli* chromosome) and it can exist in either of two alternative states. Firstly, it can exist as an autonomous self-replicating circular molecule, rather like a very small second chromosome, which normally replicates whenever the cell divides; bacteria with an autonomous F are referred to as F^+ (F plus). Secondly, in an F^+ cell a single cross-over can occur between the circular F and the circular bacterial chromosome and so recombine F into the continuity of the chromosome (figure 8.2) where it behaves exactly as if it

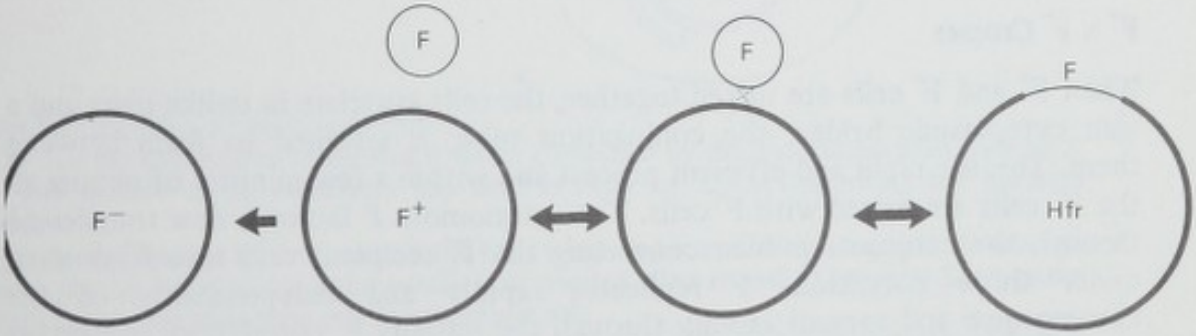


Figure 8.2 The F factor cycle. The sex or fertility factor, F, can exist as an autonomous entity in an F^+ bacterium or, by a single recombination event, it can be inserted into the continuity of the bacterial chromosome, producing an Hfr cell. F can be spontaneously lost from F^+ cells and so restore the F^- state.

were a group of bacterial genes; bacteria with an integrated F are known as Hfr (for High-frequency recombination). Hfr bacteria tend to be unstable and to revert to F^+ by a similar recombination event excising the F factor from the bacterial chromosome; F^+ bacteria can also revert by losing the F factor altogether and they become F^- (F minus) recipient cells—once F has been lost it can only be regained by the transfer of another F factor from an F^+ cell. Genetic elements such as F which are capable of existing either in a completely autonomous state or as an integral part of the bacterial chromosome are called *episomes.*

The most obvious property of the F factor is to enable donor bacteria to conjugate with recipient bacteria by the synthesis of a new surface component; this component, first identified by C. Brinton in 1964, is an appendage called an F-pilus or sex pilus. Flagellae and other surface appendages (pili) are a normal feature of many enterobacteria and it was only possible to distinguish the F-pili from the other pili on the cell surface because they are the specific sites for adsorption of the RNA phages R17 and MS2; after a population of donor bacteria has been infected with either R17 or MS2, examination under the electron microscope reveals that the phage particles have not adsorbed to the cell surface but have formed a dense coating to long (up to 20 μm) filamentous projections from the bacterial cell, the F-pili. Although many pili may be present, there is usually only one F-pilus for each F factor present in the donor cell. There can be little doubt that one of the functions of the F-pilus is to join together donor and recipient cells in mating cultures as donor ability is strictly correlated with the presence of F-pili; although the F-pilus has either an axial hole or a longitudinal groove, DNA has never been detected in a pilus and we do not know whether the genes are transferred from the donor to the recipient cell through the F-pilus itself or whether the F-pilus brings the donor and recipient cells into closer proximity, enabling the formation of another cytoplasmic bridge between them.

The F factor contains at least seven genes concerned with the formation of the F-pilus and the transfer of genetic material from donor to recipient bacterium.

$F^+ \times F^-$ Crosses

When F^+ and F^- cells are mixed together, the cells associate in unlike pairs and a thin cytoplasmic bridge, the conjugation tube, is assumed to form between them. This is a rapid and efficient process and within a few minutes of mixing all the F^+ cells are paired with F^- cells. The autonomous F factor is now transferred through the conjugation tube converting the F^- recipient cells into F^+ donors; under those conditions F replicates rapidly and independently of the chromosome and spreads rapidly through the initially F^- population, converting it to F^+. Although the F factor is transmissible at very high frequency, in $F^+ \times F^-$ crosses there is only very rare transfer of donor genetic markers (10^{-6} to 10^{-7}) and it seems probable that these rare instances of transfer are due to the occurrence of occasional Hfr variants in the otherwise F^+ population.

Very soon after the discovery of F^+ and F^- strains, William Hayes and Luigi Cavalli independently isolated donor strains which, in crosses with F^- strains produced several hundred times more genetic recombinants than any known F^+ strain. They called these strains Hfr (High-frequency recombination) and it was the subsequent analysis of these Hfr strains by Wollman, Jacob and Hayes that led to our understanding of the mating process and to the development of quite new techniques for mapping the bacterial chromosome.

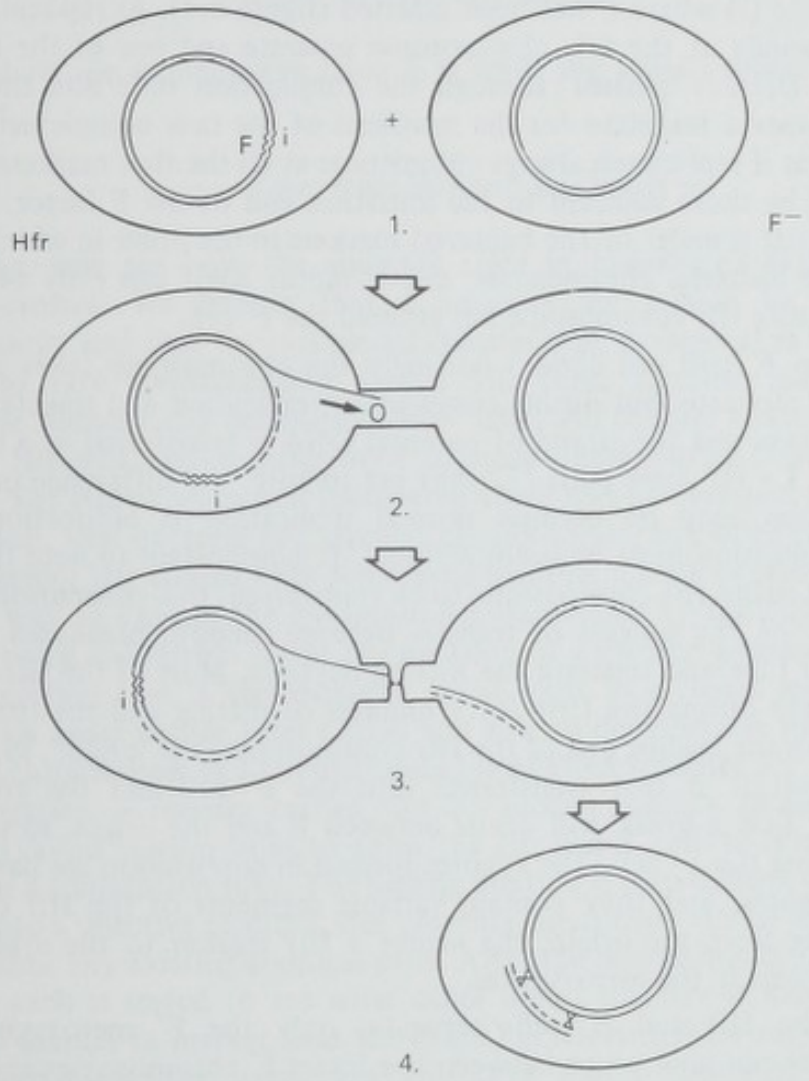


Figure 8.3 An Hfr × F^- cross in *E. coli.*

(1) An Hfr cell with an integrated F factor (indicated by the wavy lines) pairs with an F^- cell. The double circle represents the double helix of DNA comprising the bacterial chromosome.

(2) A conjugation tube forms and the Hfr chromosome commences to replicate from an initiator site (i) close to the point where F is inserted. One strand of parental DNA passes through the conjugation tube into the recipient cell.

(3) During the process of replication and transfer the mating bacteria separate and an F^- merozygote or partial diploid is formed. A complementary strand of DNA is probably synthesised in the F^- cell.

(4) Recombination occurs between the F^- chromosome and the fragment of chromosome introduced from the Hfr to produce a stable recombinant.

Hfr × F⁻ Crosses

When Hfr and F⁻ cells are mixed, conjugal pairs form exactly as in F⁺ × F⁻ crosses and there is then a partial and oriented transfer of chromosomal DNA from the Hfr into the F⁻ cell. Exactly how the Hfr chromosome is mobilised for transfer is not known, but under normal conditions transfer is accompanied by the replication of DNA and the most likely explanation is that the formation of the conjugation tube stimulates the Hfr chromosome to replicate, commencing at an initiation site (i) where F has been inserted (figure 8.3). As replication proceeds the two strands of the Hfr chromosome separate and one of the two parental strands of DNA is 'pushed' through the conjugation tube into the F⁻ cell and there becomes a template for the synthesis of the new complementary strand. Observe that if replication always commences at (i) the first markers to enter the F⁻ cell will be those adjacent to the initiation end of the F factor, followed by the sequential transfer of the bacterial markers in the order in which they occur around the bacterial chromosome; the F factor itself can only be transferred after the entire Hfr chromosome has entered the F⁻ cell.

When an *E. coli* cell divides normally the chromosome takes about thirty minutes to replicate, but during conjugation replication and transfer take about ninety minutes and the strand of parental DNA is transferred at a uniform rate of about 3.3×10^4 base pairs (12 μm) per minute; the difference in these times of replication may be because normal replication is bidirectional whereas transfer replication must be unidirectional. It is important to note that it is very rare for the entire Hfr chromosome to be transferred; this chromosome is usually severed during the process of transfer because random breakages rupture the conjugation tube and separate the mating bacteria. Most of the Hfr–F⁻ conjugal pairs separate during the first forty minutes of mating and the further a given marker from the leading end of the Hfr chromosome (the 'origin', symbolised by O), the later it is first transferred into the F⁻ cell and the greater is the probability that a break will occur between it and the origin, so preventing it from entering the F⁻ cell. The zygotes formed in conjugation are partial diploids or *merozygotes*, and they contain variable segments of the Hfr chromosome commencing from the origin; the nearer a Hfr marker to the origin the more often it appears in the merozygotes.

When the Hfr and F⁻ cells separate, only the F⁻ merozygotes survive; crossing-over can now occur between the intact F⁻ chromosomes and the partial chromosomes transferred from the Hfr cells and generate a variety of types of stable recombinant.

The recombinants from an Hfr × F⁻ cross are nearly always F⁻. This is because the F factor is at the extreme distal end of the chromosome being transferred, and so is usually prevented from entering the F⁻ cell because of spontaneous breakage of the conjugation tube. It is only when the very rare recombinants for an extreme distal marker are *selected* that the F is transferred, and these recombinants are usually found to be Hfr. In the corresponding F⁺ × F⁻ crosses, the genetic recombinants are F⁺ because F is autonomous and transferred independently of the chromosome.

When an F factor inserts into a wild type *E. coli* chromosome it appears to do so at random and in either orientation. As a result, there are very many different Hfr strains which differ both in the markers they transfer at high frequency and also as to whether the markers are transferred in a clockwise or an anticlockwise direction (see figure 8.6). For example, the Hayes strain HfrH transfers anticlockwise in the sequence

← origin-*thr-leu-ton-lac-gal*- - -

(the symbols are explained in table 7.1) while the Cavalli strain HfrC transfers clockwise

- - - *met-thr-leu-ton-lac*-origin →

We can now see how the anomaly arose in Lederberg's experiments with $F^+ \times F^-$ crosses. We believe that F^+ donors are unable to transfer their chromosomes and that the occasional transfer that is observed in $F^+ \times F^-$ crosses is due to rare spontaneously arising Hfr cells; accurate mapping will be impossible because the population of F^+ cells will contain a random variety of different Hfr cells, each transferring a different segment of its chromosome.

When Hfr strains were first discovered there was no evidence, either physical or genetical, that bacteria had circular chromosomes, but when the results from all the known Hfr strains were considered together it soon became clear that the simplest way to explain the chromosomes transferred by the different Hfrs was by each being a different linear permutation of a circular genome. This was the first suggestion that a chromosome could exist as a closed loop, but it was another six years before John Cairns was able to demonstrate the reality of the bacterial chromosome as a circular molecule of DNA (chapter 3).

Conjugation is usually carried out by mixing suitably marked Hfr and F^- strains in liquid culture so as to allow the Hfr and F^- cells to come into contact and form a conjugation tube. The mating mixture is then diluted very gently and plated onto a selective medium; this prevents any further Hfr-F^- unions but does not separate any existing conjugal pairs. After the recombinants have grown into colonies each is tested to see what other donor markers it has inherited. If a proximal marker (a marker near the origin) is selected there may be as many as one recombinant for every ten Hfr cells plated—thus the frequency of recombinants is several orders of magnitude greater than in general transduction experiments.

Mapping by Gradient of Transmission

The unique method of chromosome transfer in conjugation produces a population of merozygotes containing pieces of Hfr chromosomes all starting at the origin but terminating at random points along the chromosome so that the nearer a marker to the origin, the more frequently it is transferred into a merozygote; once a donor marker has been transferred into a recipient cell it has

about one chance in two of being recombined into the recipient chromosome. Mapping by gradient of transmission is one of two methods of analysis that depends on this unusual method of chromosome transfer.

Table 8.1 Mapping by gradient of transmission in an Hfr × F^- cross

Hfr H	thr^+	leu^+	azi^S	ton^S	lac^+	gal^+	$(\lambda)^-$	str^S	mal^+	mtl^+
F^-	*thr*	*leu*	azi^r	ton^r	*lac*	*gal*	$(\lambda)^+$	str^r	*mal*	*mtl*

selected markers	percentage frequency of unselected Hfr markers among the selected recombinants								
	thr^+ leu^+	azi^S	ton^S	lac^+	gal^+	$(\lambda)^-$	mal^+	xyl^+	mtl^+
$thr^+\ leu^+\ str^r$	–	92	73	49	31	15	0	0	0
$gal^+\ str^r$	75	75	74	74	–	84	0	0	0

After mating $thr^+\ leu^+\ str^r$ recombinants were selected by plating on minimal medium containing streptomycin (streptomycin kills the sensitive Hfr parent), and $gal^+\ str^r$ recombinants were selected by plating on minimal medium containing galactose (instead of glucose), threonine, leucine and streptomycin. $(\lambda)^+$ and $(\lambda)^-$ indicate the presence or absence of a λ prophage at the att λ locus. The other symbols are explained in table 7.1. (Data from Wollman, Jacob and Hayes, 1956.)

Let us consider a cross carried out by Wollman, Jacob and Hayes using Hfr, which transfers its chromosome in the sequence

O-*thr*-*leu*-*azi*-*ton*-*lac*-*gal*-λ-*mal*-*xyl*-*mtl*

When they selected $thr^+\ leu^+\ str^r$ recombinants by plating on minimal medium containing streptomycin they found a gradient of recovery of the unselected donor markers from *azi* to λ (table 8.1); thus any other marker within the *leu*-λ region could be assigned to a position on the chromosome by knowing how frequently it is recovered from among the recombinants. Note that thr^+ and leu^+ are the most proximal Hfr markers and they are so closely linked that in this experiment they were selected together as if they were a single gene. However, when $gal^+\ str^r$ recombinants were selected, by plating on minimal medium containing galactose (instead of glucose), threonine, leucine and streptomycin, all the markers between *thr* and *gal* were recovered at the same frequency so that no deductions could be made about their order. The reason is that when a distal marker is selected (for example, gal^+), every merozygote receives a uniform chromosome including the origin and the selected marker, so that the frequency of marker recovery is then solely dependent on the frequency of recombination—which we have just noted is the same for all markers. On the

Table 8.2 Interrupted mating in an Hfr × F⁻ cross

HfrH *thr*⁺ *leu*⁺ *azi*ˢ *ton*ˢ *lac*⁺ *gal*⁺ *str*ˢ

F⁻ *thr* *leu* *azi*ʳ *ton*ʳ *lac* *gal* *str*ʳ

time sampled (min)	constitution (per cent) of *thr*⁺ *leu*⁺ recombinants									
	untreated samples					blended samples				
	thr⁺ *leu*⁺	*azi*ˢ	*ton*ˢ	*lac*⁺	*gal*⁺	*thr*⁺ *leu*⁺	*azi*ˢ	*ton*ˢ	*lac*⁺	*gal*⁺
5	100	90	73	34	17	0	0	0	0	0
10	100	89	74	38	18	100	12	3	0	0
15	100	90	75	32	19	100	70	31	0	0
20	100	91	74	34	18	100	88	71	12	20
40	100	90	80	42	19	100	90	75	38	20

At various times after mating one sample was diluted and plated on minimal medium containing streptomycin; a second sample was vigorously blended for two minutes before plating. The recombinants were scored for the unselected markers. (Data from Jacob and Wollman, 1961.)

other hand, when a proximal marker is selected, the results are relatively uninfluenced by recombination, and we are observing only the consequences of random chromosome breakage during transfer.

The *mal, xyl* and *mtl* Hfr markers do not appear among the recombinants. The reason for this is partly because these markers are a long way from the origin and so are very rarely transferred, and partly because they are closely linked to and located distally to the *str*ˢ marker; consequently, many of the recombinants inheriting *mal, xyl* or *mtl* markers from the Hfr donor would also inherit *str*ˢ and so be killed by the streptomycin in the medium.

Mapping by Interrupted Mating

With this method, also dependent on the nature of Hfr transfer, the conjugal pairs are artificially separated by violent agitation in a high-speed blender. Firstly, because of the rapidity with which the Hfr and F⁻ cells form conjugal pairs, and secondly because of the oriented and uniform rate of chromosome transfer, a given marker will always enter the F⁻ cell at the same time after mating commences. This method establishes the time at which a marker first enters the merozygote, making it possible to construct a linkage map with the

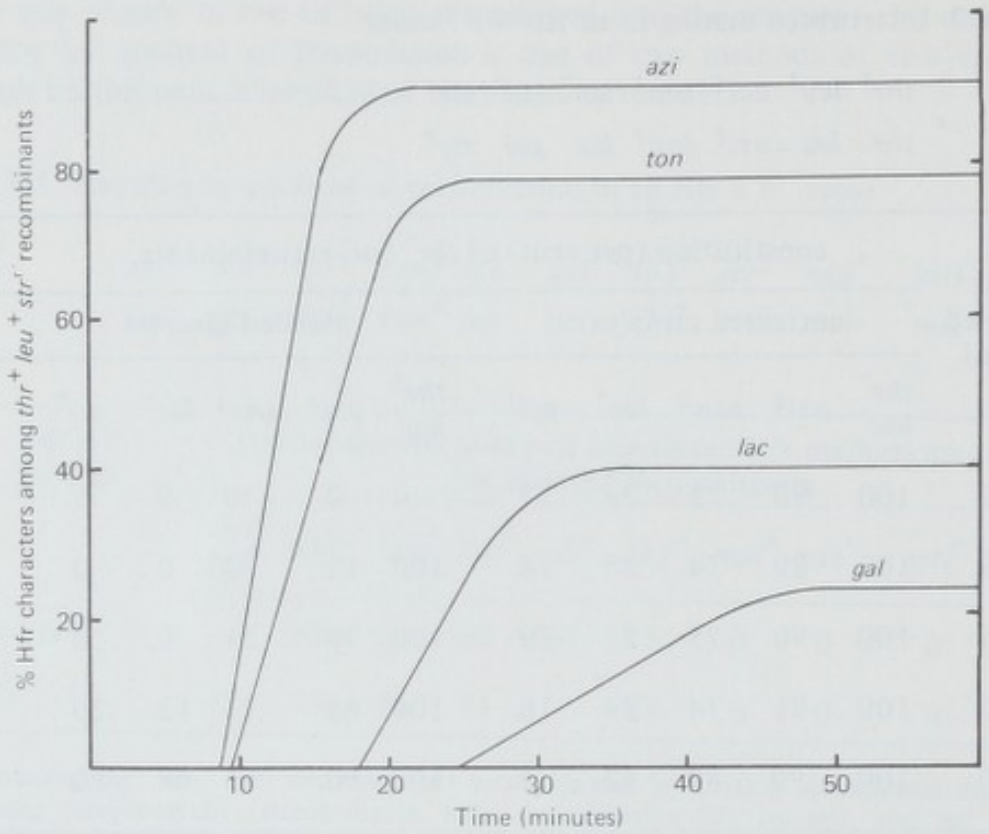


Figure 8.4 Marker recovery after interrupted mating. The Hfr markers show both different times of entry and different maximum levels of incorporation.

distances expressed in time units. The map position of any marker is easily determined by establishing the time at which the marker first appears among the recombinants. This is the most frequently used method for constructing linkage maps by conjugational analysis.

Jacob and Wollman carried out the same cross as in the last experiment, but instead of allowing mating to proceed uninterrupted they separated the conjugal pairs by mechanical agitation at various times after mating, diluted the mating mixture to prevent the reforming of conjugal pairs and plated on minimal medium containing streptomycin to select thr^+ leu^+ str^r recombinants. The frequencies of the unselected markers were determined as in the last experiment. Some of their results are shown in table 8.2 and represented graphically in figure 8.4. Observe, firstly, that there is a particular time at which each marker first enters the F^- cells, so that whenever mating is interrupted before this time the marker is absent from among the recombinants; secondly, that each marker has a different maximum level of incorporation (figure 8.4), and the earlier a marker is first transferred, the higher the plateau it reaches; this difference is due to the gradient of transmission described in the previous experiments; thirdly, that the maps constructed by gradient of transmission and by interrupted mating are in complete agreement (figure 8.5).

Over 300 gene loci have already been identified and mapped in *E. coli*, and if each locus is 1000 nucleotide pairs long (this represents an 'average' gene coding

	thr$^+$	*leu*$^+$	*azi*^{S}	*ton*^{S}	*lac*$^+$	*gal*$^+$	λ
Time of entry (min)	8	$8\frac{1}{2}$	9	11	18	25	25+
Gradient of Transmission (%)	100		90	70	45	25	15

(a) A comparison of genetic mapping by interrupted mating and by gradient of transmission.

$$0 \text{ —— } thr \underset{1.6}{\overset{\frac{1}{2}}{\text{——}}} leu \underset{1.6}{\overset{\frac{1}{2}}{\text{——}}} azi \underset{6.6}{\overset{2}{\text{——}}} ton \underset{23.1}{\overset{7}{\text{——}}} lac \underset{23.1}{\overset{7}{\text{——}}} gal \underset{1.6}{\overset{\frac{1}{2}}{\text{——}}} \lambda \text{ ——}$$

minutes
nucleotide pairs x 10^{-4}

(b) Since the chromosome is transferred at a uniform rate of 3.3×10^4 nucleotides per minute, the time intervals between markers can be converted to numbers of nucleotide pairs.

Figure 8.5 Genetic mapping of the proximal end of the HfrH chromosome

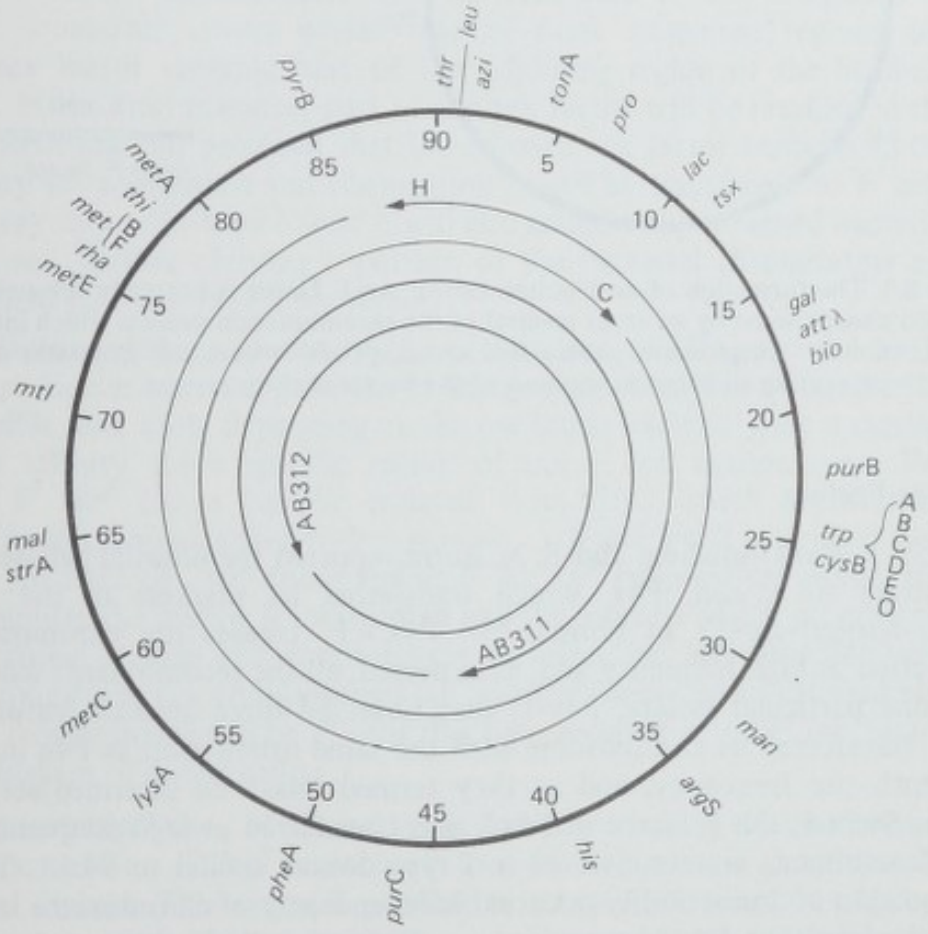


Figure 8.6 The *Escherichia coli* chromosome. The outer circle shows the map positions of some of the many known loci, and a time-scale based on an arbitrary origin at 12 o'clock. The inner circles show the points of entry and the directions of transfer of four independently isolated Hfr strains.

for a polypeptide about 330 amino acids long) this leaves 3000 loci, or 90 per cent of the chromosome, still to be identified. A map showing some of the known loci is shown in figure 8.6: this map is drawn to scale in terms of time units, and the threonine locus at 12 o'clock is arbitrarily assigned the position of 0 minutes.

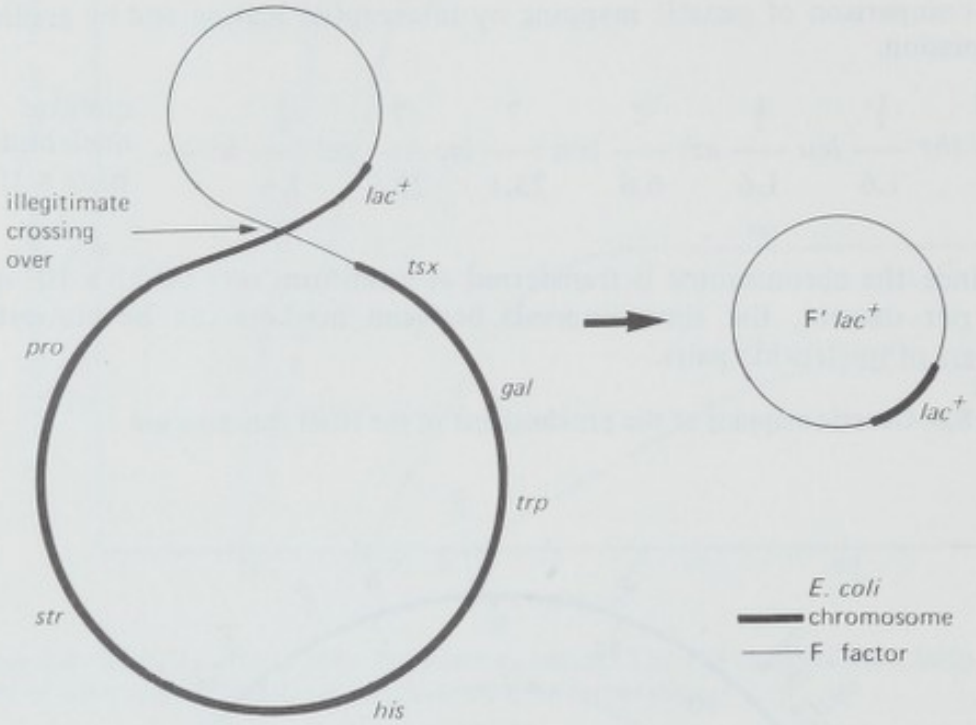


Figure 8.7 The formation of an F-prime factor. An F factor is normally released from the bacterial chromosome by an exact reversal of the recombination process which integrated it, but occasionally 'illegitimate pairing and crossing-over' occurs and generates an F-prime factor incorporating an adjacent segment of the bacterial chromosome.

F-Prime Factors

In 1959 Edward Adelberg and S. N. Burns reported the unusual behaviour of an Hfr strain of *E. coli*, P4x, which transferred its markers in the sequence O-*pro*-*leu*-*gal*-*lac*-F. In normal Hfr P4x × F⁻ crosses the chromosome was transferred at high frequency and, as expected, all the recombinants were F⁻, but with one particular isolate, P4x-1, they observed three unusual features. First, P4x-1 transferred its chromosome with the same orientation as P4x but at only one-tenth the frequency, and so they termed P4x-1 an intermediate (I) type donor. Second, the F factor of P4x-1 was transferred at *high* frequency and all the recombinants were converted to I type donors similar to P4x-1. Third, the transmission of donor ability occurred independently of chromosome transfer. It seemed clear that P4x-1 contained an F factor which alternated between a particular site on the chromosome (between *lac* and *pro*) and the autonomous state so that in a population of P4x-1 cells only 10 per cent of the cells have an integrated F and can transfer their chromosomes. Adelberg and Burns further

found that when F^- strains isolated from P4x-1 (by loss of F) were mixed with F^+ carrying a wild type F factor all the F^- cells were converted to I type donors similar to P4x-1 and not to just F^+. It is clear that not only did the sex factor from P4x-1 have an affinity for the *lac-pro* region of the *E. coli* chromosome, but also that the chromosome of P4x-1 has an affinity for a wild type sex factor. The simplest explanation was that there had been a reciprocal exchange between the original integrated sex factor of P4x and the donor chromosome itself so that the sex factor contained part of the bacterial chromosome and vice versa; thus there would be regions of homology between the P4x-1 sex factor and the bacterial chromosome and F would preferentially integrate at this site on the chromosome.

Hfr strains tend to be rather unstable and to revert to F^+ and it is thought that the normal excision process is the exact reverse of integration (figure 8.2) but that occasional errors occur, as they do in many genetic processes, and result in the formation of an F factor carrying one or more bacterial genes. We believe that 'illegitimate pairing' occurs either between a region of the integrated F and a non-homologous region of the donor chromosome (figure 8.7) or between two regions of the donor chromosome, one on each side of the integrated F; sometimes a cross-over occurs within one of these mispaired regions and generates a sex factor carrying part of the adjoining region of the bacterial chromosome. In the first instance, part of the sex factor will be retained in the bacterial chromosome but provided that the released sex factor contains all the genes necessary for replication and conjugation it will be transferred to F^- cells in the same way as a wild type F, *and* it will also transfer any attached bacterial genes. These sex factors carrying a portion of the bacterial chromosome are called *substituted* or F-*prime* (F′) *sex factors.*

These F′ factors arise spontaneously in populations of Hfr cells and an F′ carrying any particular bacterial gene can be isolated provided that a suitable Hfr strain is available, and each, depending on the particular bacterial gene it carries, will have an affinity for a specific region of the *E. coli* chromosome. For example, an F′ lac^+ factor can be isolated from Hfr2, which transfers its chromosome in the sequence O-*pro-leu-gal-lac* . . . F and has F integrated very close to lac^+. An Hfr2 lac^+ str^s donor and an F^- lac^- str^r recipient are mixed, and after thirty minutes the mating is interrupted and the mixture plated on minimal medium containing lactose and streptomycin to select lac^+ str^r recombinants. Under these conditions the chromosomal lac^+ marker cannot be transferred and the only lac^+ recombinants will be F^- cells that have received a rare F′ lac^+ factor from the population of donor cells.

One of the principal uses of F′ factors is for constructing strains that are partial diploids; thus an F′ lac^+ transferred to a F^- lac^- cell will generate a F′ lac^+/lac^- partial diploid. This makes it possible to study the interaction of genes in the same way that it is studied in higher organisms and in a way that would not otherwise be possible in bacteria—for example, it enables the relationships of dominance and recessiveness to be established and it provides another means for performing complementation tests.

The sex factor of *E. coli* can be transfered not only into other species of *Escherichia* but also into species of *Shigella* and into *Salmonella typhimurium.* Both Hfr and F′ strains of *Salmonella* have been isolated and have enabled the construction of a linkage map very similar to that of *E. coli.* Other systems of conjugation have been discovered in *Pseudomonas* and *Serratia.*

Summary of Key Points

(1) Strains of *E. coli* carrying the sex factor episome (F) are able to conjugate with F^- strains not carrying the episome. In F^+ strains, where F is autonomous, only F itself is transferred from the donor to the recipient but in Hfr strains, where F is integrated, the bacterial chromosome is transferred.

(2) The transfer of the bacterial chromosome from an Hfr to a F^- cell is an oriented process and the order of the genes along the Hfr chromosome can be determined from the order in which they enter the F^- cell. This method is very suitable for analysing the gross genetic structure of the bacterial chromosome but is of very limited use for fine structure analysis.

(3) Different Hfr strains have F integrated at different points around the bacterial chromosome and this determines which marker is the first to be transferred and the direction of transfer of the donor chromosome.

(4) When F is excised from the bacterial chromosome it sometimes incorporates into its structure one or more bacterial genes. This F-prime factor is transferred and inherited like F but always transfers the attached bacterial genes with it. F′ factors can be used to construct partial diploids which enable the functional relationships between genes to be examined.

9 Genetic Systems in Bacteriophages

The soul's dark cottage, battered and decayed, lets in new light thro' chinks that time has made.

Edmund Waller (1606-1687)

Some of the most important developments in genetics in the last fifteen years are a direct result of our being able to map the genetic material of phages; for example, not only have mapping experiments provided the most detailed fine structure analysis of any known gene (page 136) but by mapping the genes and identifying their function at the molecular level we can learn how the separate phage components are assembled into complete particles. But before we can measure recombination frequencies and construct linkage maps we must be able to estimate the total number of phages in a given population and to be able to recognise different mutants and the wild type. It is also desirable to have a selective technique so as to simplify the detection of any rare recombinants.

The Virulent Phages

Individual phage particles can be identified because they form *plaques*. When a drop of a mixture containing very many phage-sensitive bacteria and a few hundred phages is added to molten soft agar, the agar poured over the surface of a nutrient plate and allowed to solidify, and the plates incubated, the uninfected bacteria grow rapidly and form a lawn of confluent growth over the surface of the plate. But in each infected bacterium the phage replicates and lyses the cell and the several hundred phage progeny released can now infect adjacent bacteria, which in turn are lysed. Phage replication is dependent on active bacterial metabolism so that this process of lysis and infection only continues until a decline in bacterial metabolism brings it to a halt. The outcome is a circular clearing, a plaque, in the lawn of confluent bacterial growth. Since each plaque represents the initial infection of one bacterium by one phage the titre of the phage suspension can easily be calculated if we know the total number of plaques on the plate and the dilution of the phage suspension used to infect the bacteria. These plaques are also important because the first phage mutants were recognised because the plaques they formed were morphologically different from those formed by wild type phages. One very extensively analysed region includes the *r*IIA and *r*IIB genes of phage T4; these *r*II mutants (*r*apid lysis mutants) were recognised in 1946 because they formed larger and more sharply defined plaques than do wild type (r^+) particles. These mutants are

distinguishable from the wild type because the latter form small fuzzy plaques on *E. coli* strains B, S and K, whereas *r*II mutants form *r* type plaques on B, wild-type r^+ plaques on S and no plaques at all on K (see table 12.1). Other mutants used in these early experiments formed minute (*mi*) or turbid (*tu*) plaques or were host range mutants (*h*), able to grow on some strains but not on others.

Recombination in the *r*II Region of T4

If a host bacterium is simultaneously infected with two genetically different parental phages–the infection must be simultaneous as otherwise *exclusion* occurs and the DNA of the superinfecting phage is rapidly broken down by DNase and so cannot participate in recombination–then both phage chromosomes will replicate together in the infected cell and genetic recombination can sometimes occur between them and produce new combinations of the parental markers. After the infected cells have lysed the progeny phage are harvested and tested to see whether they contain any recombinants. In crosses between non-allelic mutants, such as $h^+r^- \times h^-r^+$, there may be up to 30–40 per cent recombination and the recombinants and parental types can be scored together simply by plating on a suitable indicator strain of bacteria, but in crosses between allelic mutants (*r*IIa × *r*IIb, for example) there may be less than 0.02 per cent recombination and the rare recombinants can only be detected by using a selective technique.

The most remarkable example of mapping is the work of Seymour Benzer on the *r*II region of phage T4. The first method used by Benzer was to cross all his *r*II mutants in pairs and to measure the frequency of recombination between each pair of mutant sites. *E. coli* B was doubly infected with each pair of *r*II mutants, and after lysis the progeny phages were harvested. The number of particles in the lysate was estimated by plating on *E. coli* B, on which both *r*II and r^+ phages will grow, while the number of wild-type recombinants was determined by plating on *E. coli* K, on which only the r^+ recombinants can grow. Since the double mutant class of recombinant cannot be detected, our best estimate of the *total* number of recombinants is twice the number of r^+ recombinants, and the frequency of recombination is

$$\frac{2 \times (\text{number of } r^+ \text{ phage})}{\text{total phage}} = \frac{2 \times (\text{number of phage which grow on K})}{(\text{number of phage which grow on B})} \times 100 \text{ per cent}$$

Usually the frequency of recombination was between 0.02 and 6 per cent, but by using this selective technique Benzer was able to detect recombination frequencies as low as 0.0001 per cent. From the results of his crosses he was able to construct a linkage map, arranging the mutants so that the map distances were approximately additive (figure 9.1); this is equivalent to mapping a diploid organism by a series of two-point test crosses.

As Benzer's collection of *r*II mutants grew, so this method became impracticable–to map 100 mutants by this method would necessitate nearly

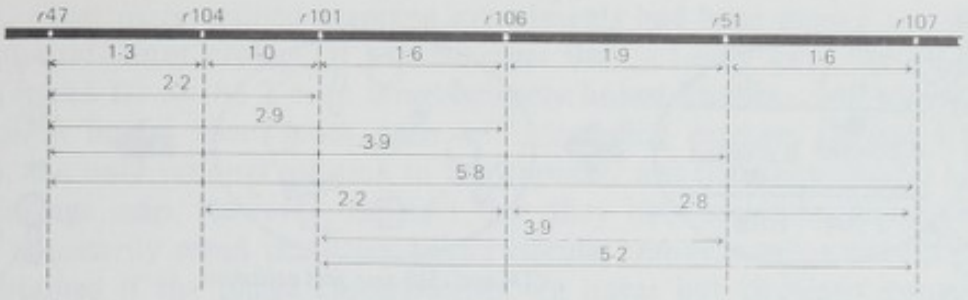


Figure 9.1 A partial linkage map of the *r*II region constructed by two-point crosses. The distances between different pairs of *r*II mutants are expressed in percentage recombination units. The distances are approximately additive and the method can be used to construct fine-scale maps of small segments of the phage chromosome (data from Benzer, 1955).

5000 crosses—and he developed and used the more rapid technique of deletion mapping (the concept of deletion mapping is explained on page 119) to locate point mutations within precisely defined segments of the genome. Altogether he mapped 2400 independently isolated *r*II mutants and identified 308 different sites within the *r*II region. Since the extreme mutant sites show 8–10 per cent recombination while the closest sites show 0.02 per cent recombination, the minimum number of sites within this region is about

$$\frac{8\text{–}10}{0.02} = 400\text{–}500$$

leaving 100–200 sites still to be detected by mutation and recombination.

Complementation in the *r*II Region

Benzer also noted that the *r*II mutants fell into two classes, *r*IIA and *r*IIB, and that any member of one class was able to complement any member of the other class. By complementation (chapter 7) we mean the ability of two mutants grown together to make good each other's defects so that, in this example, when *E. coli* K is doubly infected with an *r*IIA and an *r*IIB mutant growth and lysis occurs even though either *r*II mutant by itself is unable to grow on *E. coli* K. It is important to realise that this is not due to recombination as the progeny phage are a mixture of the original *r*IIA and *r*IIB mutants, and r^+ recombinants are not normally present (figure 9.2). From mapping experiments Benzer knew that all the *r*IIA mutants mapped at one end of the *r*II region and all the *r*IIB mutants at the other end and he correctly concluded that the *r*IIA and *r*IIB regions were separate units of function, or *cistrons*, as defined by complementation tests. This was perhaps Benzer's most important contribution as it was the first experimental definition of the gene as a unit of function and it completely destroyed the classical concept of the gene as a unit of heredity that is indivisible by either mutation or recombination.

Once we accept that the gene is the unit of function, complementation can be used as a rapid test to determine whether two mutants infecting the same cell are allelic, and mutant within the same gene, or non-allelic, and mutant in different genes. By using the complementation test an unknown mutant can be rapidly

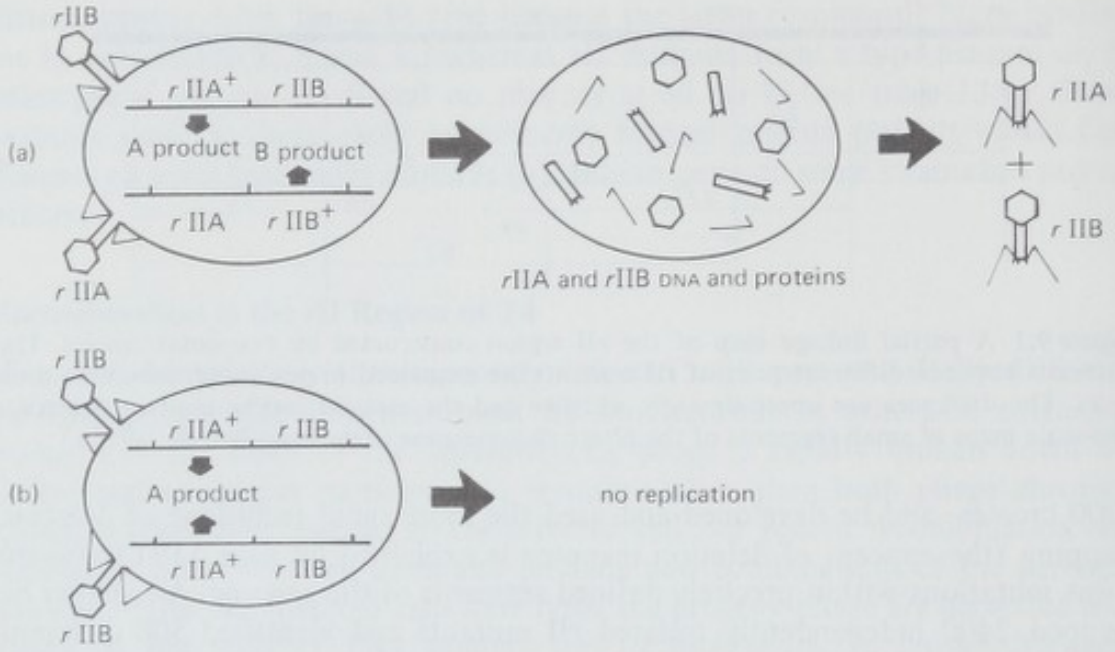


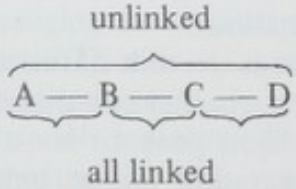
Figure 9.2 Complementation between *r*IIA and *r*IIB mutants of phage T4.

(a) Both the *r*IIA and the *r*IIB gene products are necessary before replication can occur. When *E. coli* is infected with an *r*IIA and an *r*IIB mutant the *r*IIA mutant will have an intact *r*IIB$^+$ gene and will produce *r*IIB$^+$ gene product–similarly, the *r*IIB mutant will produce *r*IIA$^+$ product. Since both gene products are present replication will occur and on lysis both *r*IIA and *r*IIB mutants will be released.

(b) When both infecting phages are mutant in the *r*IIB gene only the *r*IIA$^+$ product will be made and replication and lysis cannot occur.

assigned to a particular gene, and the location of the mutant site within that gene can be determined by standard recombinational mapping. In effect the complementation test synthesises an artificial diploid; a host cell infected by an *r*IIA$^-$ *r*IIB$^+$ and an *r*IIA$^+$ *r*IIB$^-$ phage is not functionally different from *a* + *vg* / *b* + doubly heterozygous fly of *Drosophila* which has a wild phenotype because the genetic defects on one chromosome are made good by the corresponding wild-type alleles on the other homologue. By the same criterion we can deduce that *r*IIA$^+$ is dominant over *r*IIA$^-$.

The first mapping experiments carried out in T2 indicated the presence of three separate linkage groups, but as more and more mutants were isolated and mapped it became clear that the markers could all be arranged on a single linkage map with approximately additive map distances–in other words there was only a single phage chromosome with no apparent linkage between the markers at each end of the map



By 1964, when more refined mapping experiments had been carried out using three- and four-factor crosses, it became clear that not only were the extreme markers (A and D) of the T-even phages closely linked but also that they were more closely linked than some pairs of intervening markers (B and C for example); the only rational solution to this anomaly was that these phages had a circular linkage map. However, the fact that they have a circular *linkage map* does not necessarily mean that they have a circular *chromosome*; a circular map is also obtained if the phage chromosomes are linear but circularly permuted structures, as the markers that are unlinked on one permuted chromosome may be very closely linked on a differently permuted chromosome. We have already seen from more recent studies at the molecular level that the chromosomes of the T-even phages are indeed linear and circularly permuted molecules of DNA (chapter 3).

Conditional Lethal Mutants in T4

Although large numbers of plaque morphology and host range mutants were isolated between 1946 and 1960, as genetic analysis proceeded it became increasingly evident that these mutations were all located in rather narrowly defined segments of the genome and were a highly selected group of mutants; the reason for this is simple, as most phage genes involve *indispensable functions* so that mutations within them are usually lethal and cannot be recovered. The problem of isolating mutations within and identifying the functions of these indispensable genes was not overcome until 1960, when Robert Edgar and Richard Epstein first isolated *conditional lethal* mutants of T4. These mutants are important because the lethal mutation is only expressed under a particular set of *restrictive* conditions while the function of the gene is unimpaired under a different set of *permissive* conditions. This makes it possible to propagate the mutant under permissive conditions and to study the effect of the mutation on the phage developmental process under restrictive conditions. The two classes of conditional lethal mutations are the temperature sensitive (*ts*) mutants first studied by Edgar and the amber (*am*) mutants first isolated by Epstein (the isolation of these mutants is described on page 164). The temperature sensitive mutants are able to grow at 25° but not at 42° while the amber mutants are able to grow on certain strains of *E. coli* but not on others (see pages 164 and 299). These conditional lethal mutations appear to occur more or less at random in the genome and have been found in all known T4 genes. From the point of view of physiological genetics these conditional lethal mutants have proved invaluable as under restrictive conditions the processes of phage development are arrested at characteristic stages according to the particular gene that is inactivated. The functions of the different genes can be investigated by infecting host cells under restrictive conditions and examining the lysates of these abortively infected bacteria so as to see which structural components are missing or which stage of phage component assembly is blocked. For example, a mutation in gene 34 produces phage particles complete in every way except that they have no tail fibres, while a mutation in gene 23 allows the production of

tails and tail fibres but no phage heads are synthesised (note that in recent years phage geneticists have tended to designate phage genes by numbers rather than by symbols).

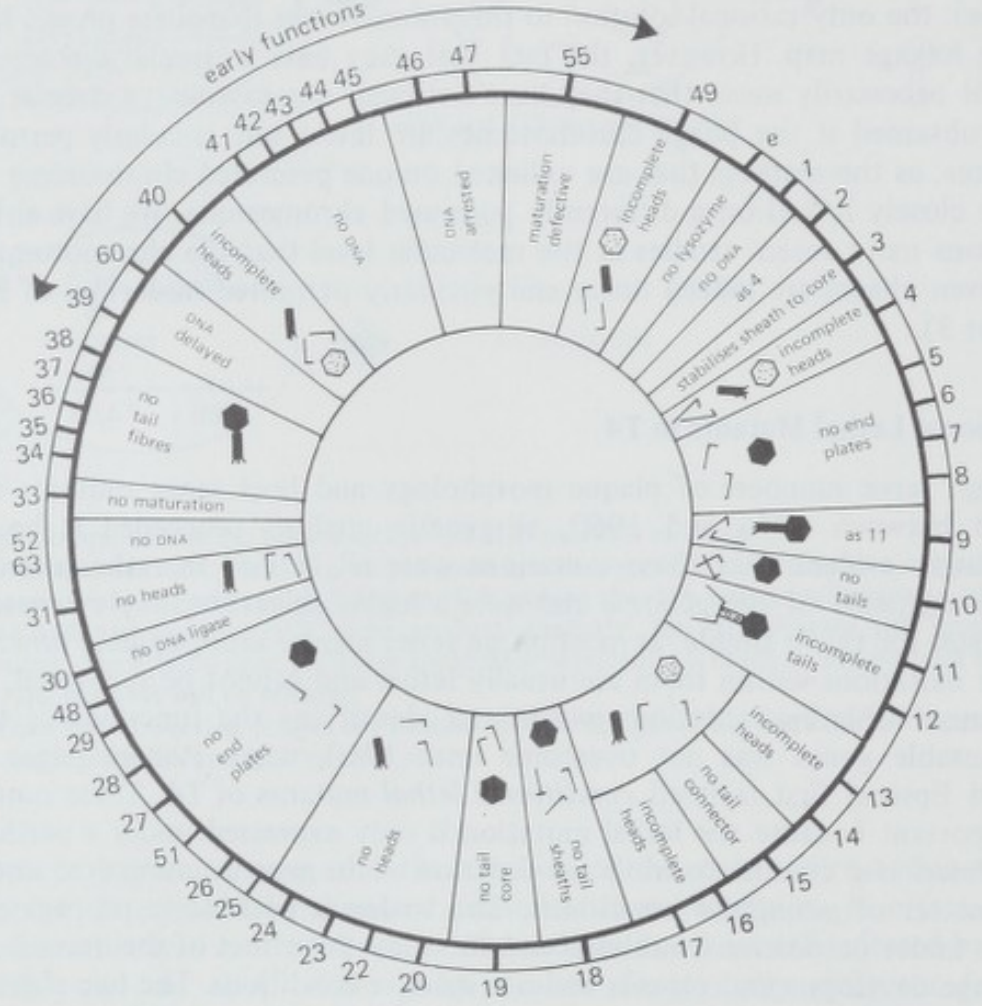


Figure 9.3 A functional map of the T4 chromosome. The outer circle represents the phage chromosome and the heavy bars the relative positions of some of the eighty genes that have been identified. By infecting host cells with conditional lethal mutants (using restrictive conditions) and examining the lysates any missing phage components can be identified. Note how genes with related functions tend to be clustered together—this is particularly so with the genes controlling the early functions, the synthesis of DNA. The picturegrams show the phage components that are present in the lysates and the legends identify the particular stage in assembly that is blocked.

The amber and temperature sensitive mutants can be assigned to particular genes by complementation tests in just the same way that *r*II mutants can be placed within either the *r*IIA or *r*IIB genes. Robert Edgar and William Wood have further shown that some pairs of mutants defective in the production of different morphological components can complement each other *in vitro*; for example, if the lysates from cells abortively infected by mutant 23 (no heads) and mutant 27 (no end plates so that the tails are incomplete) are mixed

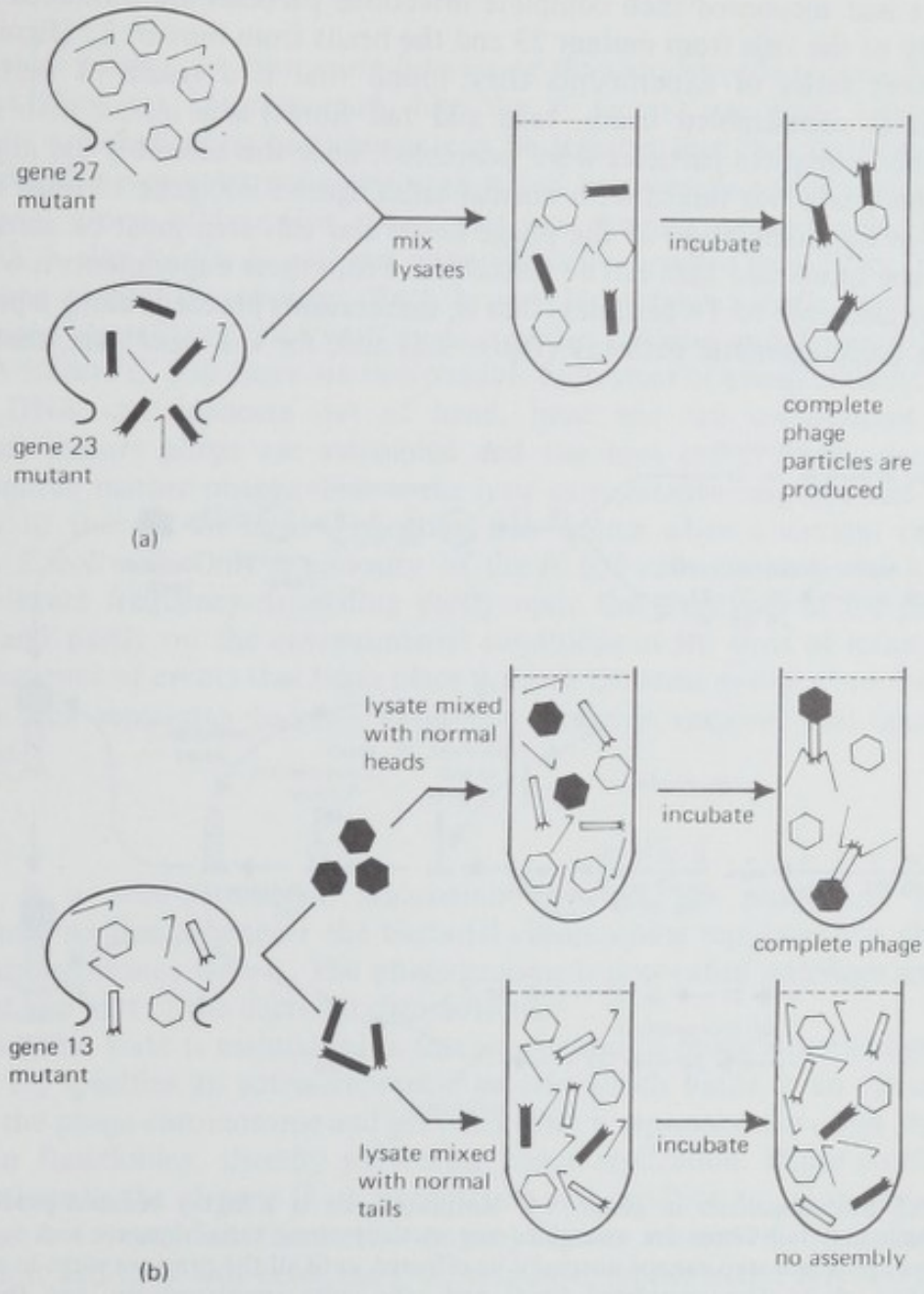


Figure 9.4 *In vitro* complementation between conditional lethal mutants of T4.

(a) A mutant in gene 23 produces tails and tail fibres but no heads while a gene 27 mutant produces heads and tail fibres but no tails. When lysates from the two abortively infected cell populations are mixed together and incubated infectious particles are produced. The gene products of the two mutants are able to complement each other *in vitro*.

(b) The lysate from cells infected with a gene 13 mutant contains what appears to be complete phage heads, tails and tail fibres, but no complete phages. When this lysate is mixed with normal (completed) heads and incubated complete phage particles are produced; no assembly takes place when the lysate is mixed with complete tails. It is clear that the heads produced by the gene 13 mutant are incomplete and defective and, further, that the heads, tails and tail fibres can only be assembled when *complete* heads are present.

together and incubated then complete infectious particles are produced by the assembly of the tails from mutant 23 and the heads from mutant 27 (figure 9.4). In another series of experiments they found that if a lysate of mutant 13 (containing unassembled heads, tails and tail fibres) was mixed with normal heads then complete particles were assembled; since this assembly did not occur when the lysate was mixed with normal tails (figure 9.4b) gene 13 must control a stage in the completion of the phage heads *and* this step must be carried out *before* the heads and tails can be assembled. From these experiments it was seen that the assembly of T4 particles (that is, maturation) proceeds along a precisely defined morphogenetic pathway (figure 9.5) and, for the most part, that a late

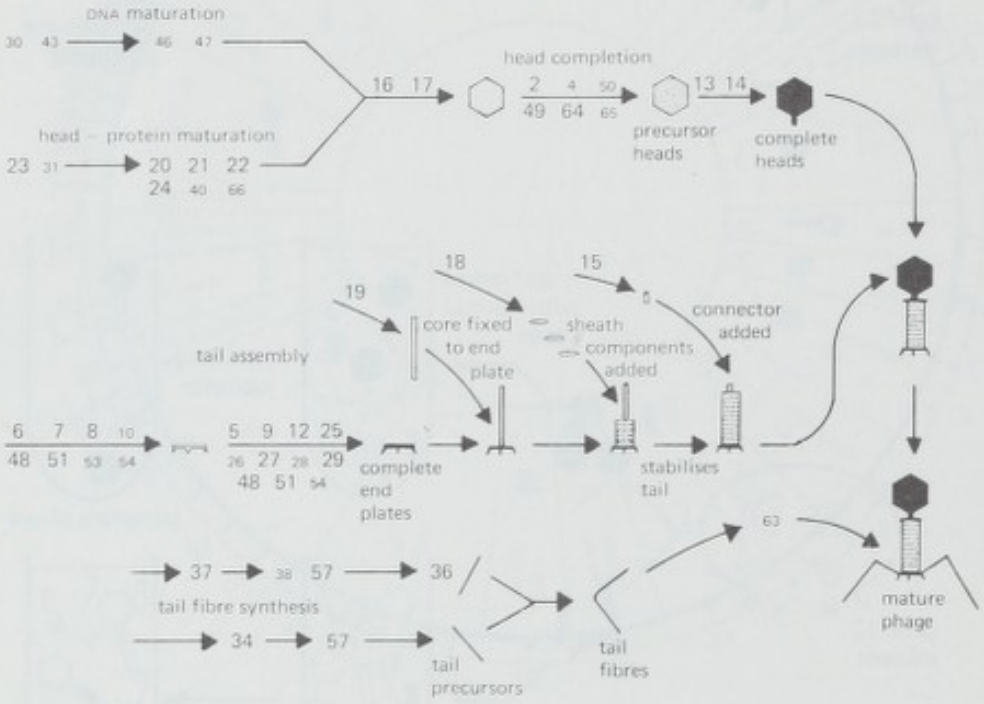


Figure 9.5 Morphogenesis in phage T4. Morphogenesis is a highly ordered process. The heads, tails and tail fibres are assembled sequentially along three separate and converging pathways and a late step cannot normally be effected until all the previous steps in assembly have taken place. The completed heads and tails unite spontaneously, and then–only then–are the tail fibres added. The genes known to encode structural proteins are shown in bold type; some of the remaining genes may also code for structural proteins but most are involved in the assembly process.

step in assembly can only be carried out if all the preceding steps have already been carried out. The genes which encode the phage structural proteins and which control the morphogenetic process are called the 'late' genes, while the genes concerned with producing the enzymes necessary for the replication of the infecting phage DNA are the 'early' genes; since the late genes can only function *after* the DNA has replicated their expression is clearly dependent upon the correct functioning of the early genes.

The Temperate Phages

The temperate phages are even more interesting than virulent phages such as T2 and T4 as they are episomes and, like the F factor, can exist either as autonomous self-replicating particles or can be inserted into the continuity of the bacterial chromosome, replicating with it and behaving just as if they were an additional group of bacterial genes. The best known temperate phage is lambda (λ). λ also infects *E. coli* and although rather smaller than T2 or T4 it has the same general morphology. Each λ particle contains a single linear and non-permuted molecule of DNA with single-stranded cohesive ends (page 40).

When λ infects *E. coli* there are two possible sequences of events. Firstly, the infecting DNA can replicate out of hand, head and tail components are synthesised, mature phage are assembled and the host cell is lysed releasing several hundred mature phages. This is the *lytic* or *vegetative* response and it is equivalent to the out of hand replication that occurs when a virulent phage infects an *E. coli* cell. Only a minority of the *E. coli* cells infected with λ are lysed, the exact frequency depending partly upon the genotypes of the phage and host and partly on the environmental conditions at the time of infection, and the sequence of events that takes place is much the same as described for T4 (page 35). The remaining bacteria enter the *lysogenic* response and become lysogenised.

Lysogeny

Lysogeny is a semi-permanent association between the phage and host chromosomes so that whenever the bacterial chromosome replicates the phage chromosome replicates with it. The phage genome is now called *prophage* and is maintained as a part of the bacterial chromosome.

The lysogenic state is maintained in this way because a phage structural gene, known as *c*I, specifies an active repressor protein which binds to an operator region on the phage chromosome and prevents (that is represses) the other phage genes from functioning, thereby preventing phage replication. Phage particles with mutations in the *c*I gene (λ *c*I mutants) do not produce any repressor and so are unable to establish themselves as prophage as whenever they infect a bacterial host cell they will enter the lytic response, replicate and lyse the cell. Normal λ particles produce rather turbid plaques because of the growth of lysogenic bacteria within the plaque but the *c*I mutants, like virulent phages, produce *c*lear type plaques.

Lysogenic cells differ from sensitive cells in two other important respects. Firstly, they are immune to *super-infection* and they cannot be lysed or lysogenised by another infecting λ particle; this is because the *c*I repressor present in the cytoplasm of every lysogenic cell can bind to the operator on the super-infecting phage chromosome and so prevent it from replicating or from becoming established as prophage. Secondly, lysogenic bacteria have the capacity to release free phage. Occasionally the prophage is released from the bacterial chromosome, replicates vegetatively and eventually lyses the host cell;

the probability of this occurring is about 10^{-5} per bacterium per generation for a typical λ lysogen. This transition from lysogeny to the lytic state can also be *induced* in up to 90 per cent of the cells in a population of λ lysogens by treating the bacteria with ultraviolet light or certain chemical compounds; this treatment destroys or inactivates the *c*I repressor and so allows the prophage to leave the bacterial chromosome and to commence replicating. The life-cycle of a temperate phage is summarised in figure 9.6.

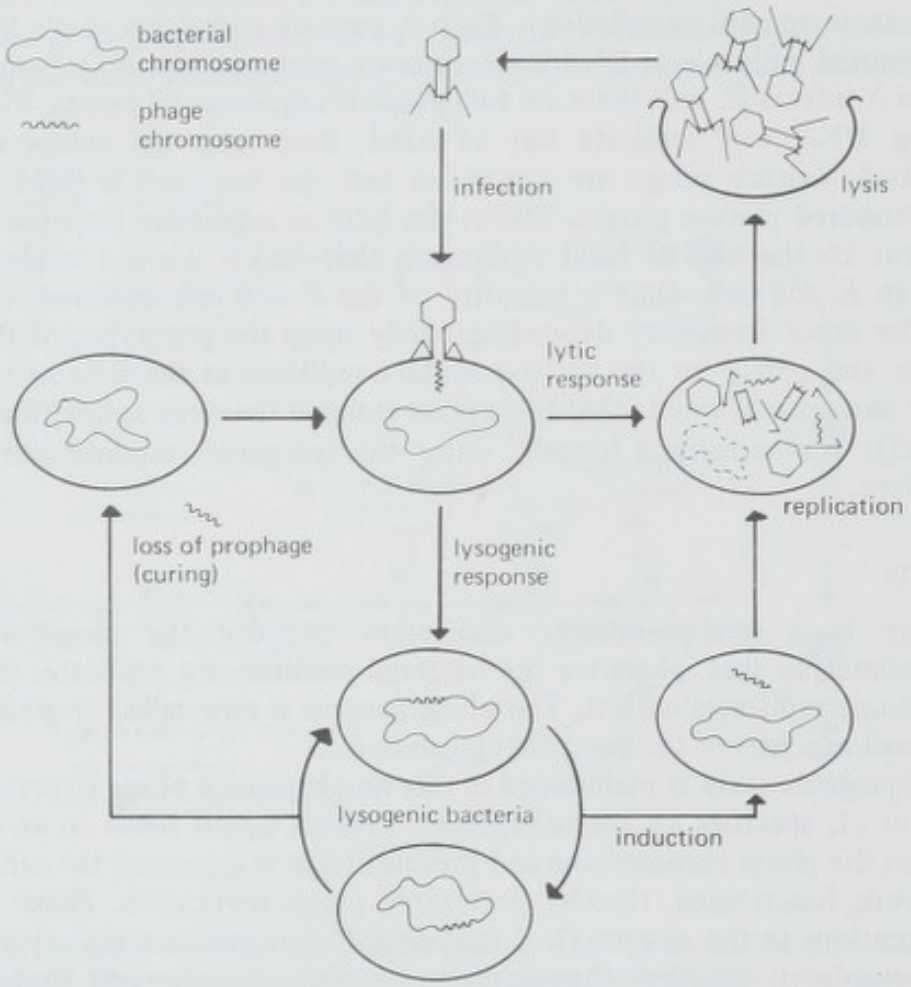


Figure 9.6 The life cycle of a temperate bacteriophage. On infection, the phage genome can either replicate out of hand and produce mature phage (the lytic response) or it can integrate into the bacterial chromosome (the lysogenic response). The integrated prophage can be induced to enter the lytic cycle or it may be spontaneously lost, segregating a sensitive bacterial cell.

λ was first discovered in 1951 by Esther Lederberg and during the following few years many mutants were isolated and mapped; these experiments, performed as for T2 and T4, revealed a single linear linkage group. At this time it was generally thought that prophage was not associated with the bacterial chromosome and was inherited as an extra-chromosomal element but several experiments soon indicated that this was not so. First, in 1953, the Lederbergs

found that in $F^+ \times F^-$ crosses between sensitive and lysogenic strains of *E. coli* the λ prophage segregates with the bacterial *gal* gene and that lysogeny could be scored among the recombinants like any other bacterial marker. Second, in 1955, E. S. Lennox demonstrated that λ prophage and *gal* could be co-transduced by phage P1 and third, in 1956, M. L. Morse discovered the restricted transduction of the *gal* genes by λ (page 148). By 1956 it seemed clear that prophage was the determinant of lysogeny and that it was located at a particular site on the bacterial chromosome. This remarkable conclusion was soon confirmed by François Jacob and Elie Wollman who used the phenomenon of *zygotic induction* to map the position of several different prophages on the *E. coli* chromosome. Zygotic induction occurs in Hfr $\times$ F^- crosses when the donor strain carries an inducible prophage and the recipient is phage sensitive; chromosome transfer proceeds normally from the donor into the recipient until the chromosome location carrying the prophage enters the non-immune F^- cell, when the prophage immediately leaves its chromosomal location, enters the vegetative state and eventually lyses the zygote releasing free phage. Not only does this result in the formation of a plaque if the cross is performed on solid

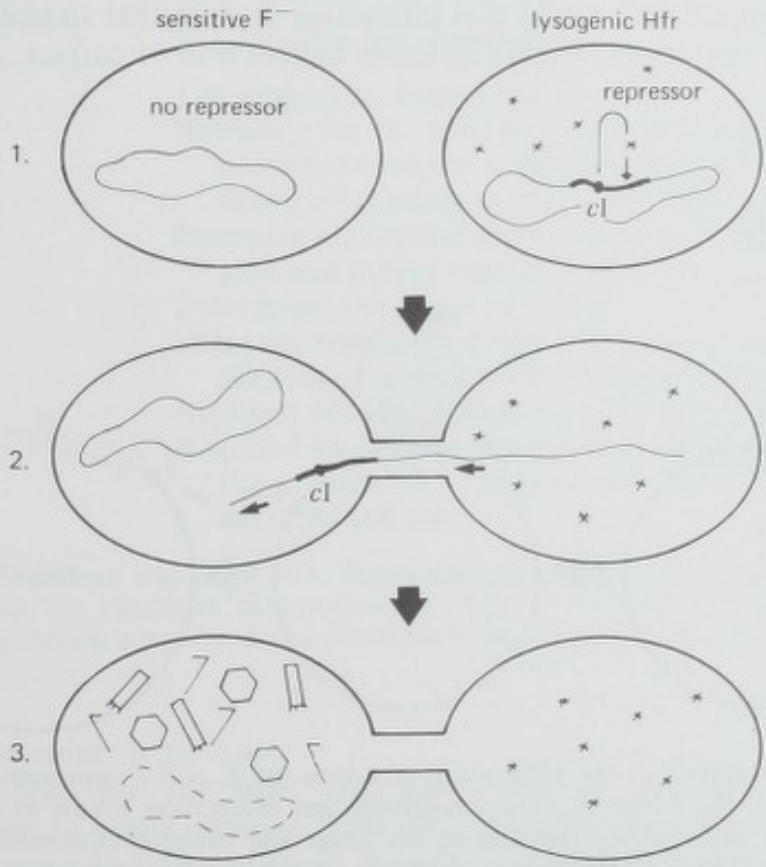


Figure 9.7 Zygotic induction. The diagrams illustrate a cross between a λ lysogenic Hfr and a sensitive F^- strain of *E. coli.* The *c*I gene of the integrated λ prophage produces a repressor which prevents the vegetative replication of the prophage (1). This repressor is not present in the non-lysogenic F^- bacterium so that as soon as the prophage is transferred into the F^- recipient (2) it is induced, leaves the bacterial chromosome, replicates (3) and eventually lyses the cell.

medium, but more importantly, since every cell inheriting the prophage is lysed no donor markers located distally to the prophage can ever be recovered among the recombinants; thus the exact location of the prophage can be mapped, either by examining the genetic constitution of the recombinants or by using interrupted mating (page 129) to determine the precise time that the prophage enters the recipient cell. We now know that zygotic induction occurs because the cytoplasm of the sensitive F^- cell does not contain any *c*I repressor so that as soon as the prophage enters this cytoplasm it is able to leave the bacterial chromosome and to replicate (figure 9.7).

Prophage Integration

For the next ten years there was considerable speculation as to whether prophage was just attached to or was actually inserted into the bacterial chromosome, and there was no significant progress towards solving this problem until 1962, when Allan Campbell proposed an elegant model to explain the insertion of prophage into the bacterial chromosome, or more generally the insertion of episomes into chromosomes. Although there is now very convincing evidence to support this model it is interesting to note that Campbell, when he first proposed the model, did not seriously believe it to be correct. According to

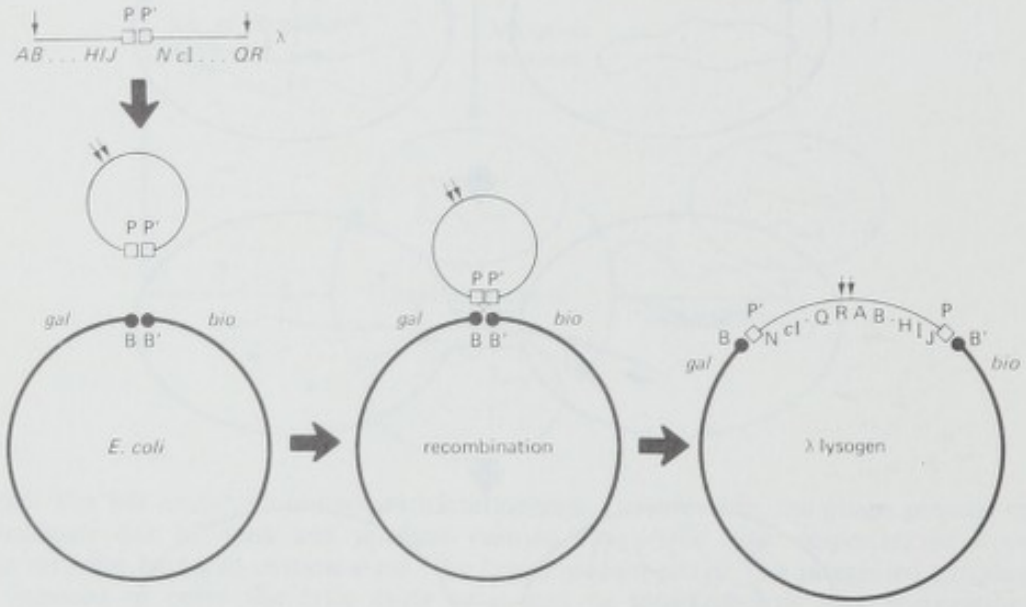


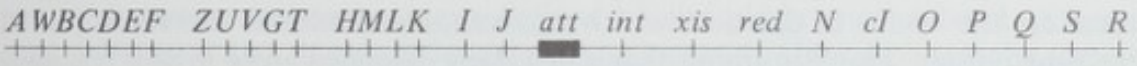
Figure 9.8 Lysogenisation–the integration of λ into the *E. coli* chromosome. On entering the bacterial cell the λ chromosome circularises and integration occurs as the result of a single cross-over between specific sites on the phage and bacterial chromosomes (att^{ϕ} and att^{B} respectively). Each of these sites appears to be duplex structure, represented P and P′ on the phage chromosome and B and B′ on the bacterial chromosome, and the actual recombination event occurs between these two pairs of sites.

Note that after integration the order of the genes along the prophage is different from the order along the infecting vegetative phage, and that recombination generates two new hybrid pairs of attachment sites.

Prophage excision occurs by the reverse process.

this model the prophage integrates by a reciprocal recombination event occurring between a special attachment site on a circular phage chromosome (att^{ϕ}) and a corresponding site on the circular bacterial chromosome (att^{B})—the result (figure 9.8) is that the phage DNA is inserted into the continuity of the bacterial chromosome; the excision of a prophage can occur by precisely the reverse process. An essential feature of this model is that the phage chromosome is circular at the time of integration, but in 1962 it was thought that the λ chromosome was linear and there was no reason to suppose that it could ever circularise. However, the following year, 1963, the λ chromosome was shown to have complementary cohesive ends (page 40) which can adhere to each other to form a circular molecule of DNA.

(a) Genetic map of λ



(b) The genes of λ

A W B C D E F	Required for head synthesis
Z U V G T H M L K I J	Required for tail synthesis
att	The phage attachment region
int and xis	Necessary for the specific recombination events that occur between the phage and bacterial attachment sites during integration and excision
red	Promotes recombination between different phage genomes during vegetative growth
cI	Determines the phage repressor
N and Q	These are regulatory genes whose products stimulate the rate of transcription of certain other phage genes
O and P	Required for the replication of phage DNA
S and R	Required for lysis of the bacterial cell; *R* determines the production of phage endolysin, which digests the bacterial cell wall.

Figure 9.9 The linkage map of phage λ. The map shows the sequence of some of the known gene loci along the vegetative chromosome of phage λ. As in *E. coli* and T4 there is a tendency for genes carrying out related functions to be clustered together.

Important points to note are:

(1) In order for the att^{ϕ} and att^{B} sites to pair together and for recombination to occur between them there must be some special relationships between their nucleotide sequences. Apart from the observation that integration always occurs between these specific regions on the phage and bacterial chromosomes, the clearest evidence for the existence of these attachment sites is that deletion mutants lacking the bacterial or phage attachment regions are unable to participate in the normal process of lysogenisation because integration can no longer occur.

(2) Since att^{ϕ} is not located within the cohesive ends the order of the genes along the integrated prophage will differ from the order on the linear vegetative chromosome—the prophage and the vegetative chromosome are cyclic permutations of each other. In the early experiments that attempted to verify the insertion hypothesis it was assumed that both prophage and vegetative phage had the same gene sequence, and this misconception resulted in much of the data being misinterpreted and so led to doubts about the correctness of the insertion hypothesis.

(3) The Campbell model requires the phage and bacterial chromosomes to be collinear. The best evidence that this is so comes from the work of Naomi Franklin on ϕ80, a close relative of λ, which integrates adjacent to the *ton-trpA* genes in *E. coli*. Among the spontaneous mutations to T1-resistance (ton^r) that occur in *E. coli* are some which also result in a requirement for tryptophan; these are the results of deletions which remove *ton* and extend into the adjacent *trp* gene cluster. Franklin found that when these deletions arose in ϕ80 lysogens many of them also extended into the adjoining ϕ80 prophage. Not only did these results confirm that the prophage and the bacterial chromosome were collinear but by determining which phage genes were missing from a series of overlapping deletion mutants (figure 9.10) Franklin was able to show, without any doubt, that the order of the genes along the prophage was a circular permutation of the vegetative map.

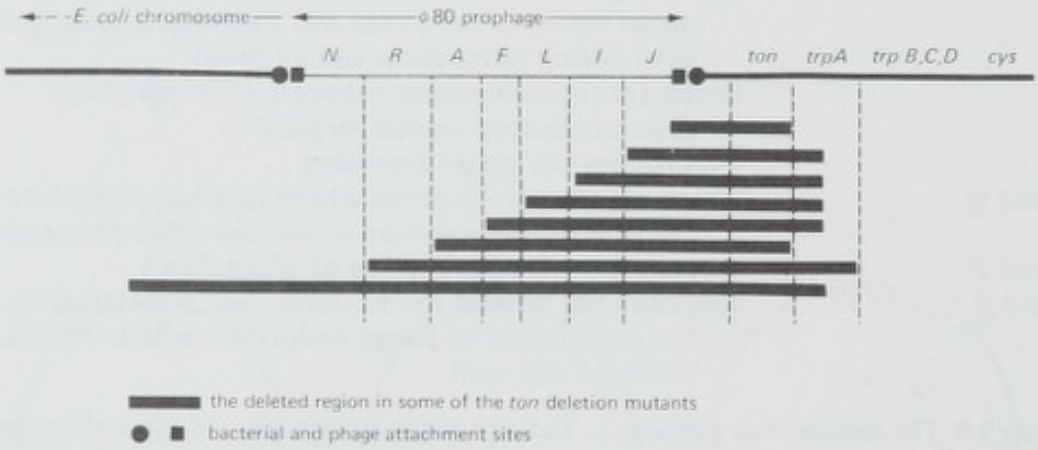


Figure 9.10 Collinearity of prophage and the bacterial chromosome. Some of the deletions involving the *E. coli ton* gene extend into the integrated prophage in ϕ80 lysogens; the missing phage genes can be arranged in a hierarchical order as the deletion of any particular phage gene always deletes *all* the more proximal phage genes but not necessarily a more distal gene. This is only expected if, as shown, the prophage and bacterial chromosome are collinear.

Restricted Transduction

In general transduction *any* marker on the bacterial chromosome can be transferred by transducing phage into a recipient bacterium, and in chapter 7 we saw that after the *lytic* infection of *Salmonella typhimurium* with phage P22 rare transducing particles are produced which contain small pieces of bacterial

DNA and no phage DNA. Phage λ can also effect transduction but the only genes that can be transduced are those on both sides of the bacterial attachment site—transduction is *restricted* to the genes in the *gal* and *bio* regions. Unlike the particles which effect general transduction the transducing λ particles arise only after the *induction* of a λ lysogen and they have part of their own DNA replaced by bacterial DNA. Because they are missing about 25 per cent of their own DNA these particles are *defective* and when they infect a sensitive bacterium they are unable to complete the replication process and so cannot give rise to infectious progeny; nevertheless they can integrate into the bacterial chromosome and can confer immunity to superinfection on the cell. After induction of a λ lysogen the prophage is normally excised from the bacterial chromosome by a single recombination event between the attachment regions but very occasionally (about 10^{-5}) an error occurs in the excision process and an *illegitimate cross-over* occurs between non-homologous regions of the phage and bacterial chromosomes (figure 9.11). If this cross-over occurs beyond *gal* (as shown in the figure)

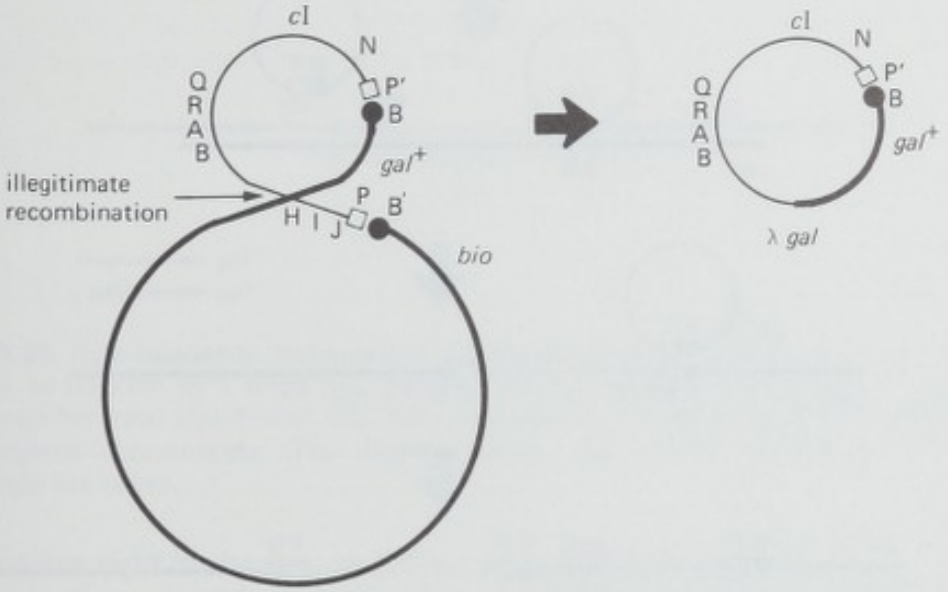


Figure 9.11 The generation of λ*gal* transducing phage. Instead of prophage excision occurring by the normal mechanism an illegitimate cross-over sometimes occurs between non-homologous regions of the phage and bacterial chromosomes—this generates a phage genome that is missing about 25 per cent of the phage genes (the HIJ region) and has instead incorporated the adjacent bacterial *gal*$^+$ genes.

Note that the λ*gal* transducing phage has a hybrid attachment site BP'.

the excised genome will have the phage genes from the right end of the phage chromosome (HIJ region) replaced by the bacterial *gal* genes; a similar cross-over occuring beyond *bio* will generate a transducing particle with the *bio* genes replacing genes from the left end of the phage chromosome. These transducing particles are referred to as λ *gal* and λ *bio* respectively.

Transduction is effected by inducing (for example) a *gal*$^+$ λ lysogen, harvesting the progeny phage and using them to infect a *gal*$^-$ *E. coli* recipient. When this mixture is plated on minimal medium containing galactose instead of

glucose about one *gal*$^+$ transductant is recovered for every 10^{-5} to 10^{-6} infecting phages (this is LFT, or low-frequency transduction). These transductants are of two types–about one third are stable *gal*$^+$, and, as in general transduction, the *gal*$^+$ gene carried by λ *gal* has been recombined into the recipient and has stably replaced *gal*$^-$. The remaining two-thirds of the transductants are unstable and persistently yield *gal*$^-$ segregants; these transductants are partial diploids (sometimes called *heterogenotes*) and in addition to the *gal*$^-$ gene on the recipient chromosome they have a *gal*$^+$ gene carried on the λ *gal* prophage. These low frequency transductions, because of the low frequency of the transducing particles, are usually carried out using a high multiplicity of infection so that every recipient bacterium infected by a defective λ *gal* particle will also have been infected by a normal λ$^+$ particle, and it is thought that λ$^+$ first integrates at *att*^{B} in the usual way immediately followed by the integration of λ *gal* at one of the 'hybrid' attachment sites produced by lysogenisation (figure 9.12).

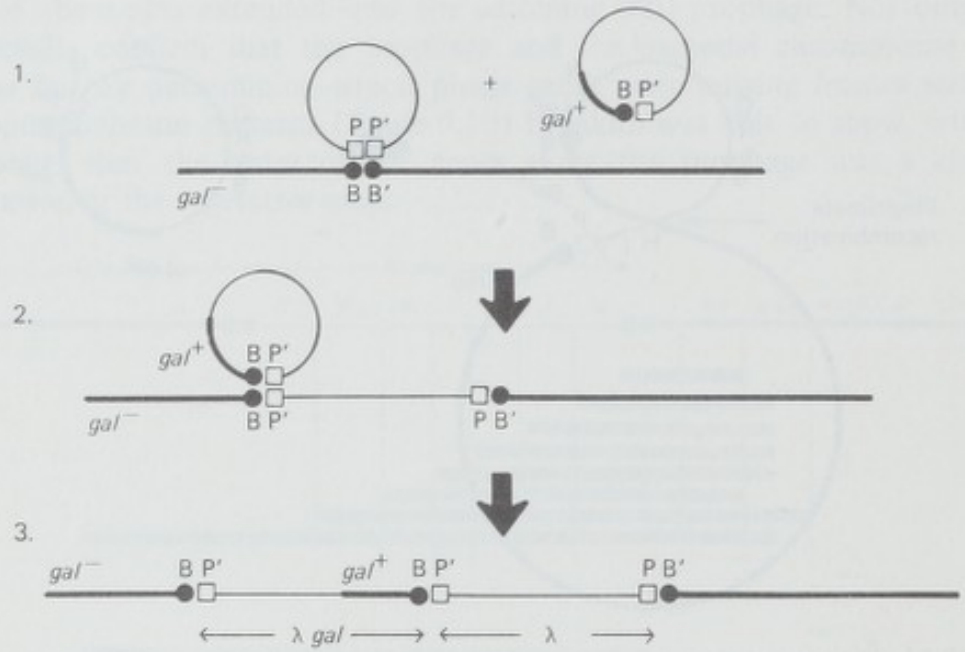


Figure 9.12 Restricted transduction by λ*gal*. When a sensitive *E. coli gal*$^-$ recipient is infected with a λ and a λ*gal* particle, as in low-frequency transduction, it is thought that the wild type λ first integrates at BB′ in the usual way (1), followed by the integration of λ*gal* at the new hybrid BP′ attachment site (2), generating a λ/λ*gal* double lysogen (3). It is possible, but less likely, that λ*gal* can integrate at PB′.

High-frequency Transduction

If this λ/λ *gal* double lysogen is now induced, the defects of λ *gal* are complemented by the corresponding genes on the wild-type prophage and on lysis equal numbers of λ$^+$ and λ *gal* particles are recovered. This lysate now contains 50 per cent transducing particles, and even when a *gal*$^-$ recipient is infected at a low multiplicity of infection there is a very high frequency of transduction (HFT, high-frequency transduction). At such a low multiplicity of

infection, double infection is rare and most *gal*$^+$ transductants will only have been infected by one λ *gal* particle; under these circumstances the λ *gal* prophage is recombined into the recipient *gal* gene because of the homology between the two *gal* regions (figure 9.13). It appears that the hybrid attachment site of λ *gal* only recombines with the normal bacterial attachment site with a very low efficiency—thus λ *gal* can only integrate at *att*^{B} if a wild-type λ integrates there at the same time and generates further pairs of hybrid attachment sites which can then recombine with the hybrid attachment site on λ *gal*, as shown in figure 9.12.

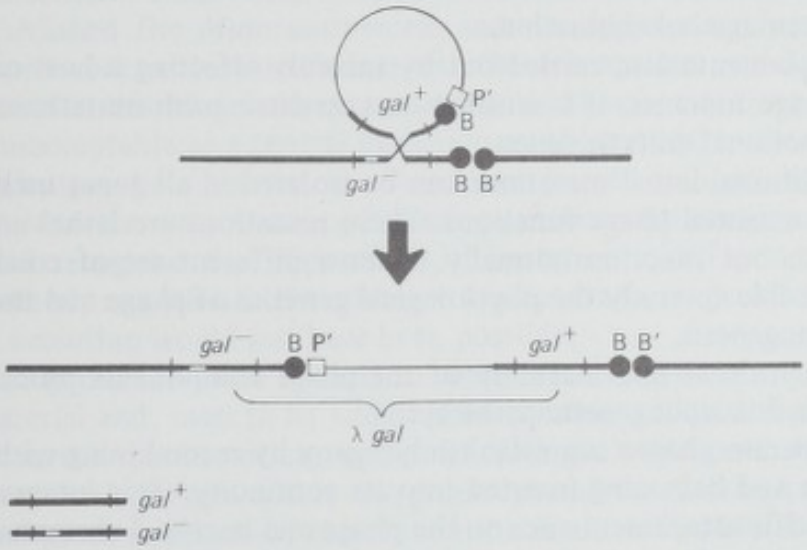


Figure 9.13 High-frequency transduction. In high-frequency transduction a *gal*$^-$ recipient cell may be infected by a single λ*gal* particle; the λ*gal* appears to be unable to integrate at the normal bacterial attachment site (BB′) and instead is recombined into the *gal* region of the recipient chromosome. The diagram shows one of the possible ways that this integration can occur.

If we are right in the way we believe transducing phage is generated, it should not normally be possible to cotransduce *gal* and *bio*, as the transducting particles will contain either the bacterial genes to the left of *att*^{B}, or those to the right of it, but never both. The fact that the cotransduction of *gal* and *bio* does not occur (except by the use of special genetic tricks) is circumstantial evidence for the correctness of our assumptions.

Other Temperate Phages

Not all prophages are like λ and φ80 with specific sites of attachment on the bacterial chromosome. Thus the *Shigella* phage P2, which can also lysogenise some strains of *E. coli*, has a strong preference for a particular site near *his* but it can also integrate at several other sites. The general transducing phage P1 is not known to attach to the bacterial chromosome at all and it is probable that P1 prophage exists as a plasmid (a plasmid is a dispensable piece of DNA which can function and replicate autonomously; note that while all episomes are plasmids,

not all plasmids are episomes). The *S. typhimurium* phage P22, like λ has a specific attachment site within the *pro* gene cluster and this phage, in addition to effecting general transduction, can also participate in the restricted transduction of the *pro* genes.

Summary of Key Points

(1) Recombination occurs between different phage mutants and linkage maps can be constructed with the distances between the markers expressed in units of percentage recombination.

(2) Complementation, carried out by mixedly infecting a host cell with two different phage mutants, is a sensitive test to distinguish mutations that are in different functional units or genes.

(3) Conditional lethal mutations can be isolated in all genes including those that specify essential phage functions. These mutations are lethal under one set of conditions but function normally under a different set of conditions; this makes it possible to study the physiological genetics of phage and the process of phage morphogenesis.

(4) The synthesis and assembly of the phage components proceeds along a strictly defined morphogenetic pathway.

(5) Temperate phages can establish lysogeny by recombining with a bacterial chromosome and becoming inserted into its continuity. This integration occurs between specific attachment sites on the phage and bacterial chromosomes.

(6) When prophage is induced to leave the bacterial chromosome it occasionally incorporates into its chromosome some of the *adjacent* bacterial genes. The resulting phage particles can transduce these bacterial genes into sensitive recipient bacteria; this is restricted transduction. The formation of these transducing phages is very similar to the formation of F-prime factors (see chapter 8).

10 Mutation

Change is not made without inconvenience, even from worse to better.
Richard Hooker (*c.* 1554–1600)

In 1901 the Dutch botanist Hugo de Vries, later to be one of the rediscoverers of Mendel, published *Die Mutationstheorie* and concluded, largely as a result of twenty years' work with *Antirrhinum* and *Oenothera,* that evolution occurs by means of sudden discrete changes or, as he called them, *mutations.* Although his ideas are unacceptable as a general evolutionary theory, and many of the sudden heritable changes he observed in *Oenothera* turned out to be due not to mutation but to the reassortment of existing genetic variation by recombination, de Vries did recognise the importance of mutation as a fundamental genetic process. Indeed, mutation is the ultimate source of all new genetic variation and without it evolution would not have been possible.

Mutation (from the Latin *mutare,* to change) is any heritable alteration in the genetic material and, used in its widest sense, includes such diverse phenomena as changes in the number of chromosomes, changes in the gross structure of the chromosomes and changes within the genes themselves. Although changes in the number and gross structure of the chromosomes are of considerable importance to the evolutionary geneticist and to the plant breeder, they have made little contribution to our understanding of the fine structure and function of the genes and, apart from a brief indication of the range of these *chromosomal* mutations, they will not be considered further, and our attention will be focused on gene or *point* mutation.

Changes in Chromosome Number–Polyploids

Polyploid individuals or populations are the result of a change in chromosome number by the loss or acquisition of one or more chromosomes or complete sets of chromosomes. Most polyploids are much less fertile than their diploid parents; for example, in a triploid, containing three complete sets of chromosomes, the chromosomes are unable to associate in their correct pairs at meiosis, they fail to segregate regularly and the gametes produced have unbalanced collections of chromosomes. For this reason polyploidy is mainly confined to species that do not need to reproduce bisexually and so has been of little importance in evolution within the animal kingdom. It has been of much greater importance in the evolution of plants, since not only are many polyploids larger and more vigorous than the corresponding diploids but the reduced fertility of newly arisen polyploids is largely offset by the ability of many plants to reproduce vegetatively. For this same reason plant breeders have worked extensively to develop new polyploid varieties of commercially

important species; for example, the most vigorous and high-yielding species of wheat (*Triticum*) are thought to be tetraploids (four sets of chromosomes) or hexaploids (six sets of chromosomes).

Changes in Chromosome Structure–Rearrangements

These are the result of the rearrangement of existing genetic material following chromosome breakage. Rearrangements have been detected in a wide range of organisms, including bacteria and fungi, but they have been most extensively studied in *Drosophila*, where they can be easily recognised by the altered pattern of banding along the polytene chromosomes (figure 10.1) and by the way in

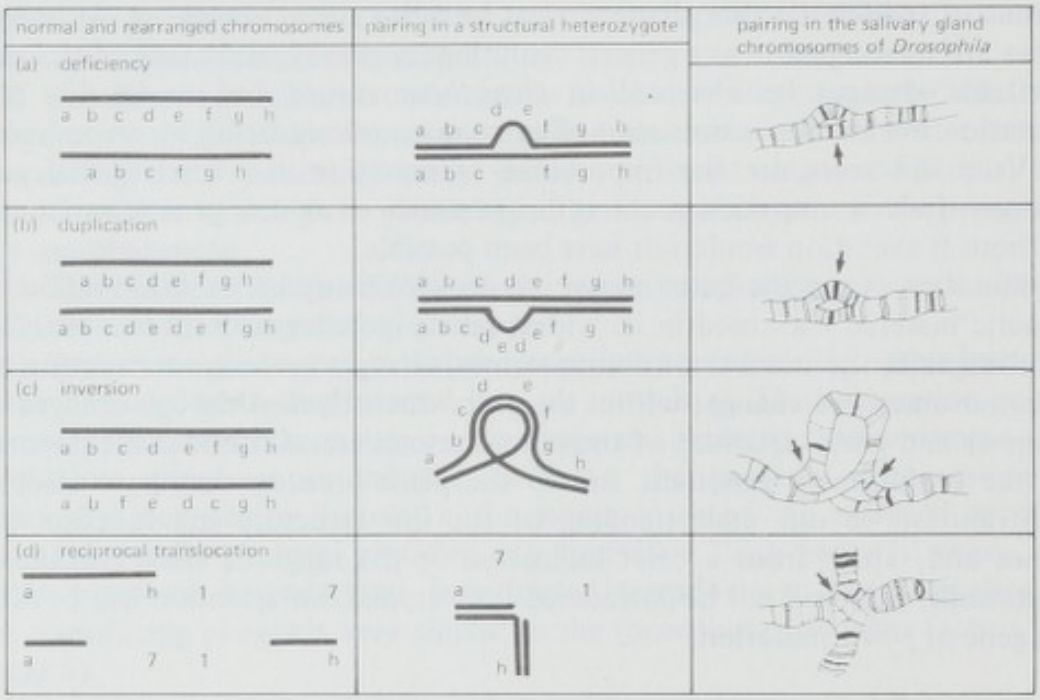


Figure 10.1 Chromosome rearrangements. In each row the upper chromosome(s) has the standard (normal) sequence of genetic information while the lower has the rearranged sequence. At meiotic prophase in a structural heterozygote the normal and rearranged chromosomes pair along their length and, as far as possible, all the homologous regions are synapsed. In a deficiency (a) and duplication (b) heterozygote there is a segment on one chromosome which has no homologous region with which to pair and there is a short unsynapsed region. In an inversion heterozygote (c) the only way that complete pairing can occur is by the formation of an inversion loop. In a translocation heterozygote (d), the two normal and the two interchanged chromosomes are synapsed in a cross-like configuration.

The drawings at the extreme right show the appearances of the normal and rearranged segments in the salivary gland of *Drosophila* structural heterozygotes.

which the homologous chromosomes synapse in structural heterozygotes (that is, individuals with one normal and one rearranged chromosome). The four most important types of rearrangement are:

(i) *Deletion or deficiency:* a segment of chromosome is missing. If the piece lost is large the change may be lethal (figure 10.1a).

(ii) *Duplication:* a segment of a chromosome is represented twice. The well-known 'Bar-eye' mutation in *Drosophila melanogaster*, which reduces the eye to a narrow slit and behaves as a Mendelian dominant, is due to the duplication of one band (16A) on the X-chromosome (figure 10.1b, see also page 306).

(iii) *Inversion:* a segment of a chromosome is inverted in relation to the rest of the chromosome so that the order of the genes along this segment is reversed. In inversion heterozygotes, mitosis is normal but at meiosis the only way that the normal and inverted chromosomes can pair all along their length is by the formation of an inversion loop (figure 10.1c). An important consequence of this is that an inversion loop effectively acts to suppress crossing-over, and recombination within an inversion loop is never observed; the explanation is that whenever a chiasma occurs within a loop the recombinant chromosomes are either dicentric (with two centromeres) or acentric (with no centromere) and the resulting gametes are inviable.

(iv) *Reciprocal translocation:* Translocation occurs when a fragment of one chromosome becomes attached to a non-homologous chromosome, and in a reciprocal translocation there is an exchange of fragments between two non-homologous chromosomes. Mitosis in a translocation heterozygote is normal but at meiotic prophase the two translocated and the two normal chromosomes associate together in a cross-like structure (figure 10.1d) and the only viable gametes contain either the two translocated or the two normal chromosomes; thus inversion heterozygotes are semi-sterile.

In structural homozygotes meiosis is normal, because end-to-end pairing can occur, and the rearrangements can only be detected by the altered linkage relationships between the markers on the normal and rearranged segments.

Gene or Point Mutation

By definition a point mutation must always occur *within* a gene (if it extends from one gene into another it ceases to be a point mutation and becomes a multisite mutation) and it should be the smallest possible change in the structure of the genetic material that is detectable as a mutation, but in practice this definition is too restrictive since it is seldom possible to distinguish between a change involving a single nucleotide pair (the smallest possible change) and a larger change involving a *small* number of adjacent nucleotides. Thus the distinction between a minute chromosomal rearrangement and a 'point' mutation is somewhat artificial and it is more convenient to regard a point mutation as the addition, deletion or substitution of one to a few nucleotides within a gene.

Point mutations (figure 10.2) can be classified as either *base-substitution* mutations, due to the replacement or substitution of one base pair for another so that the total number of base pairs in the genetic message remains unchanged, or *frameshift* mutations, due to the addition or deletion of one or two base pairs from the DNA.

In protein synthesis the genetic information (the nucleotide sequence) along the double-stranded molecule of DNA is first transferred onto a single strand of messenger RNA, which then acts as a template for the assembly of a polypeptide (figure 1.5). Each group of three nucleotides (codon) on the mRNA is recognised by a specific transfer-RNA molecule, which, in turn, attaches the correct amino acid onto the growing polypeptide chain, thus an alteration in the nucleotide sequence along the DNA can result in a changed amino-acid sequence along the polypeptide specified by that gene. The base-substitution mutations can be either samesense, missense or nonsense mutations, according to the way in which they affect the amino-acid sequence in the polypeptide (for convenience it is simplest to assume that these mutations have occurred in a structural gene coding for a polypeptide rather than in a gene carrying out a regulatory function). A *samesense* mutation (figure 10.2b) is not usually

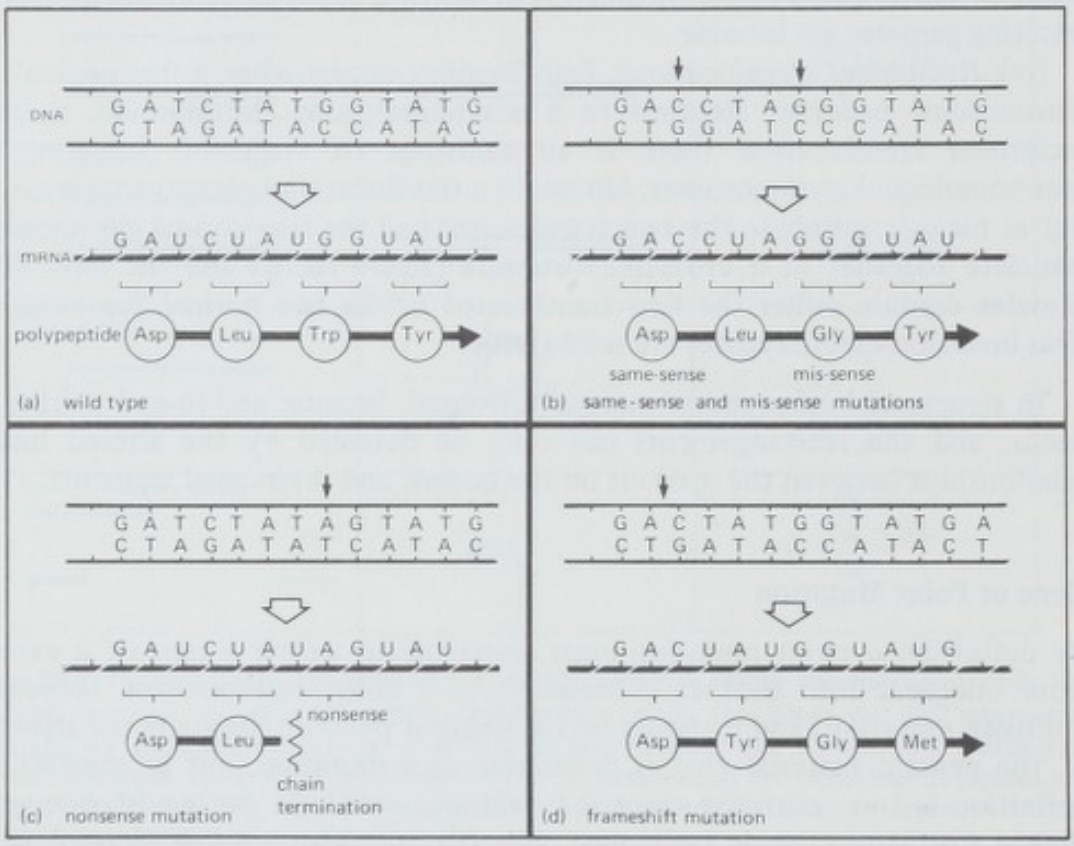


Figure 10.2 Point mutations

(a) The nucleotide sequences of a small segment of a wild-type gene and of the corresponding molecule of mRNA, and the amino-acid sequence of the polypeptide specified by this segment.

(b) A samesense mutation has occurred in the aspartic acid codon and a missense mutation in the tryptophan codon.

(c) A nonsense mutation has occurred in the tryptophan codon.

(d) A frameshift mutation has occurred in the aspartic acid codon.

Note that the mRNA code must always be read from a fixed starting point, since otherwise three different messages would be read, depending on whether reading starts with the sequences GAU.CUA . . . or AUC.UAU . . . or UCU.AUG . . .

The small arrows indicate the position at which the mutations have occurred.

recognisable, for although the structure of the DNA is altered the same amino acid is still inserted into the polypeptide. For example, aspartic acid is coded by either of the triplets GAU or GAC; a base-pair substitution in the DNA that converts a mRNA codon from GAU to GAC will be unrecognisable, since both the GAU and GAC codons are recognised by a transfer RNA molecule carrying aspartic acid, and this amino acid will be inserted at the corresponding position in the polypeptides in both the wild-type and the samesense mutant. A *missense* mutation is a base-pair substitution in the DNA that alters a mRNA codon so that it now encodes a different amino acid; that is to say, the altered codon is recognised by a completely different tRNA molecule. In figure 10.2b a T-A base pair in the tryptophan codon has been replaced by a G-C pair and the new codon in the mRNA (GGG) is now recognised by a tRNA-carrying glycine. Since the amino-acid sequence of a polypeptide determines its secondary, tertiary and quaternary structure, many missense mutations (but not by any means all) will result in the complete or partial inactivation of a protein and the expression of a mutant phenotype. *Nonsense* mutations (figure 10.2c) are base-pair substitutions that change a mRNA codon into one of the three nonsense codons, UAG, UAA or UGA. When mutation generates a nonsense codon in the middle of a gene, peptide chain elongation stops as soon as translation reaches the nonsense codon and only an inactive polypeptide fragment is produced. *Frameshift mutations* (figure 10.2d) add or delete a base pair from the message, and their effect is to shift the reading frame of the code so that all the codons from the one containing the extra or missing base to the end of the message are read out of phase. Any polypeptide produced will either contain a hopelessly incorrect amino acid sequence or else will be only a fragment because of the generation of a nonsense codon in the out of phase part of the message. These mutations are further discussed in chapters 12, 13 and 18.

Types of Mutant Available for Study

Mutations can be classified according to their most conspicuous effect on the phenotype as:

(i) *Visible mutations,* where the effect of the mutation is seen in the phenotype of the organism, such as vestigial wing or ebony body in *Drosophila.*

(ii) *Biochemical mutations,* leading to the loss of a specific biochemical function, such as the inability of certain bacterial or fungal mutants to synthesise a specific amino acid.

(iii) *Lethal mutations,* inevitably causing death of the organism. Lethal mutations are relatively frequent, but this is only to be expected as most organisms are well adapted to their environment and many gene mutations might be expected to be harmful. Although lethal mutations cannot be studied in haploid organisms such as bacteria, recessive lethal mutations have been extensively used in mutational studies with *Drosophila.*

(iv) *Conditional lethal mutations,* viable under one set of conditions but lethal under other conditions; for example, the temperature-sensitive mutants of

phage T4 are able to grow normally and to form plaques on an *E. coli* host at 25°C but are unable to do so at 42°C.

Although this is a convenient descriptive classification, it must be remembered that these are not mutually exclusive classes; for example, since most genes carry out specific biochemical processes, nearly all gene mutations are in reality biochemical mutations.

Spontaneous Mutation

Mutation in Higher Organisms

Many different types of organism are used in mutation experiments, and each has its own special advantages and disadvantages. At one extreme are the micro-organisms which have the advantages of a very short generation time (sometimes only twenty minutes), the availability of screening and selective techniques for the isolation of mutants and the measurement of mutation rates and the possibility of carrying out fine-scale genetic analysis. At the other extreme are the sexually differentiated organisms, such as *Drosophila*, which offer not only the advantages of conventional genetic analysis but also the opportunity for correlating genetic analysis with cytological examination.

Although selective techniques for isolating new mutant strains are not available in *Drosophila*, very many mutants have been isolated by simply collecting the occasional variants arising spontaneously in pure-breeding laboratory strains. Wild populations are another source of new mutant strains; since gene mutations are occurring spontaneously all the time, even though at a very low rate, wild populations carry many recessive mutant genes in a heterozygous condition. The Russian geneticist Sergei Tshetwerikoff collected 239 wild *Drosophila* flies and after carrying out suitable breeding tests he isolated from them a number of visibly abnormal flies; after further tests he was able to show that thirty-two of these were new visibly recessive mutants. This simple experiment also emphasises a fundamental rule of mutation research—no new variant can be considered to be due to genetic mutation until the altered phenotype has been shown to segregate in accordance with Mendel's principles; some of the variants could be due to non-inheritable environmental variation.

Every gene has its own characteristic rate of mutation (table 10.1), some genes mutating more frequently than others. It must also be remembered that a gene can show very different rates of *forward* mutation (a change from a wild-type to a mutant allele) and *back* mutation or *reversion* (a change from a mutant to a wild-type allele). We expect this as if a wild-type gene is, say, 1000 nucleotide pairs long, any change involving any one of the 1000 nucleotide sites within the gene is a potential forward mutation; on the other hand, a true back-mutation to the wild-type allele can only occur by reversing the forward mutation and so involves a specific change at one particular site within the gene. Thus the forward and back mutation rates may differ by several orders of magnitude.

In animals only mutations occurring in the germ line can be transmitted to the progeny and mutation frequency is expressed as the number of occurrences

Table 10.1 Comparative mutation rates in different organisms

Organism and character		Mutation rate
Man		
haemophilia		3×10^{-5} per gamete per generation
Zea mays		
waxy endosperm	$Wx \rightarrow wx$	0
non-purple aleurone	$Pr \rightarrow pr$	1×10^{-5}
shrunken endosperm	$Sh \rightarrow sh$	1×10^{-6}
colourless aleurone	$R \rightarrow r$	5×10^{-4}
Drosophila melanogaster		
white eye	$w^{+} \rightarrow w$	3×10^{-5}
yellow body	$y^{+} \rightarrow y$	12×10^{-5}
Neurospora crassa		
adenine independence	$ad3 \rightarrow ad^{+}$	$*4 \times 10^{-8}$ per conidium
Escherichia coli		
T1 resistance	$ton^{s} \rightarrow ton^{r}$	2×10^{-8} per bacterium per generation
histidine requirement	$his^{+} \rightarrow his^{-}$	2×10^{-6}
histidine independence	$his^{-} \rightarrow his^{+}$	$*1 \times 10^{-9}$
lactose fermentation	$lac^{-} \rightarrow lac^{+}$	$*2 \times 10^{-7}$
Phage T4		
*r*II locus	SM32 $\rightarrow r^{+}$	$*2 \times 10^{-7}$ per phage per replication
	UV199 $\rightarrow r^{+}$	$*8 \times 10^{-8}$
	SM1 $\rightarrow r^{+}$	$*5 \times 10^{-11}$

Note that the frequency of forward mutations, which can occur at any one of many sites within a gene, is generally higher than the frequency of reverse mutations (*), which can only occur by a change at a particular site within the gene. *r*II SM32 is a frameshift mutant, UV199 is due to a G–C → A–T transition and SM1 is probably due to a transversion.

per gamete per generation—in *Drosophila* a 'typical' gene has a forward mutation rate of 10^{-4} to 10^{-5} per gamete per generation. It is clear that to measure accurately the rate of spontaneous mutation of a specific gene is a very laborious process and many ingenious techniques have been devised to enable mutation rates to be more easily measured, mainly so as to be able to assess the effects of various treatments on mutation rates. One method for measuring mutation rates in *Drosophila* is the Muller-5 method devised by Hermann J. Muller. This technique (figure 10.3) does not measure the mutation rate of any one particular gene but measures the rate of mutation to sex-linked recessive lethals at a large

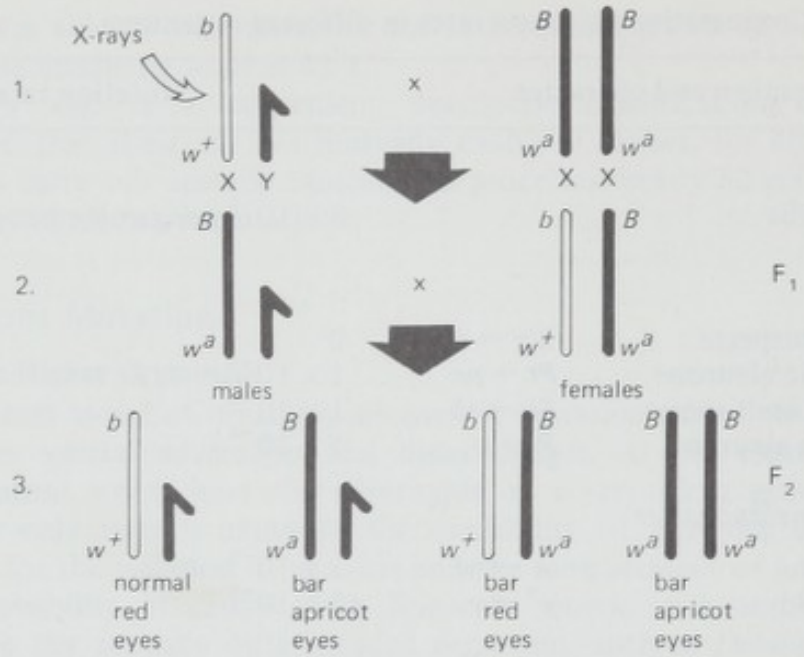


Figure 10.3 The Muller-5 method for detecting sex-linked recessive lethal mutations.

(1) Wild-type males are X-irradiated and mated with Muller-5 females. These females are homozygous for the Muller-5 chromosome; this X-chromosome carries the dominant Bar-eye gene (*B*), the recessive apricot eye gene (w^a) and an inversion to suppress crossing over in the heterozygous F_1 females.

(2) The F_1 females are individually mated to F_1 males. Each F_1 female represents one irradiated X chromosome (unshaded).

(3) If no sex-linked recessive lethal mutation has been induced there will be four types of F_2 progeny in a 1 : 1 : 1 : 1 ratio; but if the treated X chromosome does carry a recessive lethal mutation there will be *no* wild-type males in the F_2 culture and twice as many females as males; each such culture represents one mutation to an X-linked recessive lethal.

number (possibly 1000) loci on the X-chromosome; thus the frequency of mutants is very much higher than if mutations at just one specific locus were being measured. Other and rather similar techniques enable the detection of autosomal recessive lethal and visible sex-linked mutations.

Mutation in Bacteria

Although it was widely recognised that spontaneous mutation has played an important role in the origin of new genetic variation in higher organisms, bacteria were regarded as a quite different form of life and bacteriologists were slow to accept that spontaneous mutation also occurred in bacteria. Nevertheless, two things were clear: firstly, that bacteria had hereditary attributes, since a single bacterium would give rise to a clone of descendents in every way similar to the original parent cell; and secondly that, since true breeding variants could appear in otherwise pure cultures, bacteria must show genetic variation; thus if a large number of bacteria (say 2×10^8) from a sensitive culture of *Escherichia coli* are plated on agar in the presence of an excess of a virulent phage, such as T1, most of the bacteria will be lysed and killed but a few will survive, divide, and eventually grow into a colony of T1-resistant bacteria. Most bacteriologists accepted the theory of *acquired*

hereditary immunity and thought that the bacteria *only* acquired resistance in the *presence* of the phage—that is to say, the environment directs specific hereditary changes in some of the cells of the population. According to the alternative theory of *spontaneous mutation*, mutations to phage resistance occur all the time in any culture of sensitive bacteria (albeit at a very low frequency) and the phage particles *select* these already existing mutants by killing all the sensitive bacteria. Thus in order to demonstrate spontaneous mutation in bacteria it was necessary to show that the phage-resistant bacteria were present in the culture *before* exposure to the phage (the selective agent). This was first achieved by Salvador Luria and Max Delbrück using a statistical test, called the *fluctuation test*, to analyse their results; they were also able to calculate that the rate of mutation from T1-sensitivity to T1-resistance was about $2–3 \times 10^8$ mutations per bacterium per generation. Their paper, published in 1943, was a clear-cut demonstration of spontaneous mutation in bacteria and marked the birth of bacterial genetics.

A few years later Harold Newcombe, using a simple respreading test, was independently able to confirm this conclusion (figure 10.4). He spread about

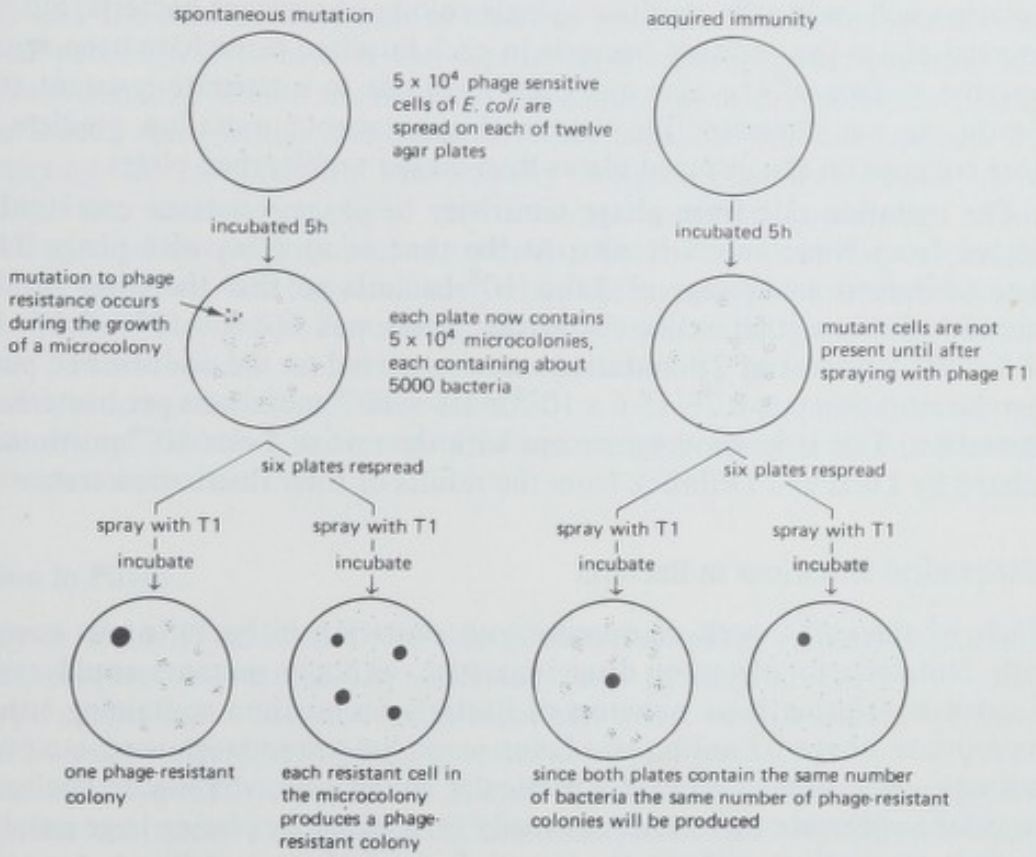


Figure 10.4 The Newcombe respreading experiment. According to the theory of spontaneous mutation (left) mutations to phage resistance are occurring all the time and independently of the presence of phage; on the other hand the theory of acquired immunity (right) states that mutations to phage resistance only occur in the presence of the phage. The simple experiment shown above enabled Harold Newcombe to distinguish between these theories.

5×10^4 sensitive cells of *E. coli* on each of twelve plates and incubated the plates for five hours. During this time the bacteria went through approximately 12.3 division cycles, so that each bacterium plated grew into a microcolony of about $2^{12.3}$, or 5100, cells. At this stage six of the plates were respread so as to distribute the bacteria in these microcolonies uniformly over their surfaces and *all* the plates were sprayed with T1 phage and incubated overnight. On examination, the six undisturbed plates contained a total of twenty-eight, and the respread plates a total of 353, T1-resistant colonies. These results are inconsistent with the theory of acquired hereditary immunity since, at the time of spraying with phage T1, both the undisturbed and the respread plates contained the same *total* numbers of bacteria and exposure to T1 should have produced the same numbers of resistant bacteria on each and so, after the final incubation, both series of plates should have yielded similar numbers of resistant colonies. On the other hand, according to the theory of spontaneous mutation, mutations to phage resistance occur randomly during growth, so that if the sample plated contained a resistant mutant, or if a mutation to resistance takes place during the growth of a microcolony, then each mutant cell would grow into a localised clone of T1-resistant cells. On the undisturbed plates each mutation will eventually produce a single colony of resistant bacteria, but on the respread plates the resistant bacteria in each localised clone have been separated over the surface of the agar and each gives rise to a separate resistant colony. Clearly, as was observed, the theory of spontaneous mutation predicts many more colonies on the respread plates than on the undisturbed plates.

The mutation rate from phage sensitivity to phage resistance can also be calculated from Newcombe's results. At the time of spraying with phage T1 each plate contained an average of 2.6×10^8 bacteria so that the total number of bacterial divisions that occurred on the plates was $6(2.6 \times 10^8 - 5.1 \times 10^4) = 15.6 \times 10^8$. A total of 28 mutations were observed on the undisturbed plates so that the mutation rate is $28/15.6 \times 10^{-8} = 1.8 \times 10^{-8}$ mutations per bacterium per generation. This is in good agreement with the rate of $2\text{–}3 \times 10^{-8}$ previously calculated by Luria and Delbrück from the results of their fluctuation test.

Biochemical Mutations in Bacteria

Much of the early work on spontaneous mutation in bacteria was concerned with mutation to phage or drug resistance, as these mutants could easily be selected by plating large numbers of bacteria on medium containing either the appropriate phage or antibiotic. Later work has extensively used auxotrophic mutants, only able to grow if a particular amino acid, vitamin or nucleotide is provided, as reverse mutations can easily be selected by plating large numbers of bacteria on medium without the growth factor (minimal medium). Auxotrophic mutants, first isolated in 1941 by Beadle and Tatum using the mould *Neurospora crassa,* were first isolated in *E. coli* in 1944 by Edward Tatum, but since these mutants are characterised by their *inability* to grow on minimal medium it was not possible to isolate and study large numbers of auxotrophic mutants until a *screening* technique was developed. Since these mutants are so

extensively used in genetic and biochemical research we will outline one method used to isolate them.

A culture of bacteria is grown and, usually, exposed to a mutagenic agent to increase the proportion of mutant cells (see page 165). The treated bacteria are then suspended in complete medium and incubated so that the cells go through one or two division cycles. This is necessary for two reasons: firstly because the bacteria may have been multinucleate at the time of treatment so that the mutant nucleus must segregate from the non-mutant nuclei before the mutation can be expressed; and secondly because it may take several divisions before the enzyme specified by the now mutant gene disappears from the cytoplasm. The concentration of mutants in the culture can now be increased by penicillin screening, a method that makes use of the fact that the antibiotic penicillin acts by killing *dividing* cells, having no effect on non-dividing cells. At this stage the bacteria are centrifuged down, washed, resuspended in liquid minimal medium containing a lethal concentration of penicillin, and incubated. The prototrophs continue to divide and are selectively killed while the auxotrophs, unable to divide because the growth factor is not present, will survive. For this screening procedure to be effective the concentration of bacteria must be low (about 10^7 per ml) and the time of treatment as short as possible. This is because the end result of penicillin treatment is the rupture of the prototrophic bacteria. This will release metabolites into the medium, the auxotrophs will commence division and be killed; there is also the possibility that penicillin-resistant mutants will arise among the prototrophs and rapidly outgrow the auxotrophs. After screening the culture is plated on minimal medium containing a low concentration of nutrients (enriched medium). On this medium the prototrophs will divide and grow into large colonies, while the auxotrophic mutants, because of the limited amounts of nutrient available, will only grow into minute colonies. These minute colonies can be picked off, grown and tested to determine which growth factor they require.

This method enables a wide variety of auxotrophic mutants to be recovered with comparative ease.

Mutation in Phage

As early as 1936, F. M. Burnet was able to show that phage variants exist which produce plaques morphologically different from those produced by ordinary wild-type phage, but it was not for another ten years, when mutants of phages T2 and T4 were isolated, that phage genetics got under way.

When wild type T2 is plated on *E. coli*, the resulting plaques are small and have a clear centre surrounded by a diffuse turbid halo. In 1946, Alfred Hershey noted an unusual plaque which was much larger and had a sharply defined outline; when he isolated phage from this plaque and replated them on *E. coli* he found they produced only large sharply defined plaques—in other words the original abnormal plaque had been produced by a true-breeding phage mutant and he designated the wild type and mutant genes r^+ and r (r standing for *r*apid lysis). Another type of true-breeding variant was the host-range mutant (h), first

isolated by Salvador E. Luria in 1945. These mutants can grow on phage-resistant bacteria so that, for example, T2*h* mutants can grow on T2-resistant strains of *E. coli*. These two classes of mutant roughly correspond to the visible mutations in higher organisms and affect observable characters (for example, plaque morphology) or specific non-essential functions (for example, host-range properties).

More important are the two classes of conditional lethal mutations, the temperature-sensitive (*ts*) mutants first studied by R. S. Edgar and the amber (*am*) mutants first isolated by R. H. Epstein. The temperature-sensitive mutants are generally thought to be *missense* mutants containing a base pair substitution; this changes the primary structure of the protein specified in such a way that it is only able to take on and retain a functional secondary, tertiary and quaternary structure at the permissive temperature (usually 25° C) while at higher restrictive temperatures (42° C) the protein is denatured and non-functional. Temperature-sensitive mutants are isolated by seeding plates of bacteria with several hundred phages, incubating the plates at 25° for a few hours until small plaques have formed and then transferring the plates to 42° (figure 10.5). The wild-type

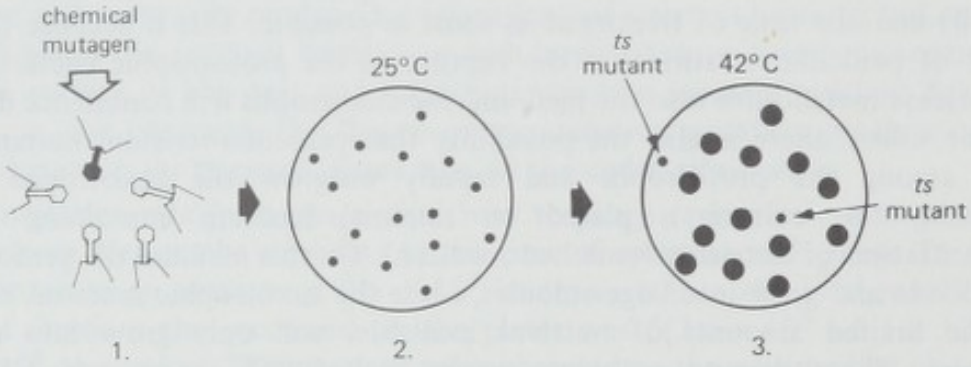


Figure 10.5 The isolation of temperature-sensitive phage mutants. Phage particles are treated with a chemical mutagen (1) and plated on a bacterial host. The plates are incubated at 25° C until small plaques have formed (2) and then transferred to 42° C. The wild-type plaques continue to increase in size but any plaques formed by temperature-sensitive mutants remain small (3).

plaques continue to increase in size but the plaques formed by the temperature-sensitive mutants remain small as these phages can no longer reproduce; these small plaques can be picked off and the phages grown at the permissive temperature. The amber mutants are *nonsense* mutants and, although unable to grow on a wild type *E. coli* host, they can grow on strains carrying a mutation able to suppress the nonsense codon; these strains can suppress nonsense mutations because mutation has occurred within a gene coding for a species of tRNA (page 299) changing its structure in such a way that it can now read the amber codon as sense; translation now continues to the end of the mutant phage gene as if it were wild type.

Altogether over eighty genes have been identified in T4 by the use of conditional lethal mutations. The chromosome of T4 is about 1.5×10^5 base pairs long so that if an 'average' gene is 900 base pairs long the chromosome

should contain about 150 genes—it would seem that over half the possible T4 genes have been identified. By the same methods seven genes have been identified in the small single-stranded DNA phage S13, a close relative of X174. The DNA of S13 is only about 5000 nucleotides long, sufficient to code for 5–6 'average' genes, so it would seem likely that all the genes of S13 have been identified.

Table 10.2 X-ray induction of sex-linked recessive lethals in *Drosophila melanogaster*

Time exposed to X-rays (min)	Experiment C1		All experiments		Frequency of lethal mutations (per cent)	Increase in mutation rate due to irradiation
	males tested	lethals detected	males tested	lethals detected		
0	198	0	6016	5	0.08	–
24	676	49	741	59	7.90	x 9.5
48	772	89	1177	143	12.10	x 14.6

H. J. Muller irradiated males for twenty-four and forty-eight minutes at a constant distance from an X-ray tube. The table shows the results of one particular experiment (C1), the combined data for all experiments and the increase in mutation rate due to the X-ray treatment. The recessive lethals were detected by crosses similar to those shown in figure 10.3.

Induced Mutation

In 1927, H. J. Muller proved that the mutation rate in *Drosophila melanogaster* could be significantly increased by treating sperm with X-rays (table 10.2). This outstanding discovery, first reported at the Fifth International Congress of Genetics held in Berlin, was the first demonstration that mutations could be induced. Although the techniques used by Muller were mainly designed to measure the rate of mutation to sex-linked recessives he was also able to detect some visible mutants, and while some of these occurred at previously undetected gene loci others were allelic to existing visible mutations. All the evidence suggested that these induced mutations were essentially similar to those occurring spontaneously and it seemed clear that induced mutation, like spontaneous mutation, is a random process. At the same time as Muller was carrying out his experiments, Lewis J. Stadler was trying to produce X-ray and radium-induced mutations in barley; this he succeeded in doing, but because barley has a much longer life cycle than *Drosophila* his results were not available until 1928, the year after Muller's results were published. Many earlier attempts had been made to induce mutations by radiations and by other treatments, but they were unsuccessful because the genetic techniques used were not adequate to demonstrate the comparatively small increases in mutation rate.

These discoveries were important in several ways. Not only did they provide a new and more rapid way for isolating the many mutant strains required for fruitful genetic analysis and stimulate investigation into the nature of the mutation process itself, but they showed that high-energy radiation is dangerous not only to the exposed individual but also to his descendants.

Radiation and Mutation

The most important type of radiation to which living organisms are exposed are the ionising radiations, such as X-rays, gamma rays and alpha, beta and other fast moving particles. Ionising radiations occur naturally, both as cosmic rays and as emissions from radioactive substances in the soil and rock formations, and artificially, as a result of nuclear disintegrations produced under man's control, such as the explosion of nuclear devices. Although X-rays and gamma rays are electromagnetic waves while alpha and beta radiations are subatomic particles, their chemical and biological effects are very similar. The alpha particles have a very high energy but they have very little power of penetration and so they are of little importance unless they arise from radioactive materials already inside the body—they are then very destructive because of their high energy. The beta particles are fast-moving electrons with variable energies and powers of penetrations. As they may travel up to 1 cm in soft tissues, they have proved useful in the treatment of tumours but, like alpha particles, when administered externally they only cause superficial damage. Gamma rays and X-rays are high energy electromagnetic radiations with very considerable penetrating powers; high energy gamma rays can traverse the whole human body and so can produce extreme radiation damage.

These radiations vary not only in their penetrating powers but also in their ability to cause ionisation. When these radiations pass through matter some of their energy is adsorbed by the constituent atoms causing them to release high energy electrons (beta particles); these atoms are said to be *ionised*. The released electron moves off with a very high energy and in turn causes the ionisation of other atoms lying along its path; each high-energy electron produces about 230 ionisations along its track and it is these ionisations that cause the principal biological effects of irradiation.

If an organism is exposed to irradiation, one of several things may happen. If the dose is sufficiently high the organism may die, or if it survives there may be severe radiation damage; in mammals this occurs as sickness, burns, loss of hair, leukaemia, etc. With a lower sub-lethal dose there may be no visible damage but the organism may become sterile, either permanently or temporarily. But *any* radiation, no matter how small the dose, can cause genetic damage or mutation. Sometimes these mutations will be in a somatic cell and will produce a mosaic—for example, irradiation of *Drosophila* larvae can produce a rare adult male with a white sector in an otherwise normal red eye; during eye development a mutation of w^+ (red eye) to w (white eye) occurs and all the eye cells derived from this mutant cell will lack red pigment while the other eye cells derived from the unmutated cells will have the pigment and be red. In a like way

irradiation of a seedling can produce a flower with coloured petals on one side and white petals on the other. However, unless these organisms can be vegetatively propagated these mutations cannot be transmitted to the next generation and so are of little interest to the geneticist—what is of concern is the mutations which occur in the reproductive cells and so can be transmitted to the progeny.

We are still largely ignorant of the ways in which ionising radiations cause genetic damage but it is possible that they can alter the structure of chromosomes in two ways: directly, by quanta of energy, which hit the chromosome, rather like bullets hitting a target; and indirectly, by ionisation, which produces chemical changes in the cell, which in turn cause anomalies when the chromosomes replicate. In general, dividing cells are much more sensitive to radiation than non-dividing cells. This explains why, for example, the larvae of *Drosophila* containing many dividing cells are much more sensitive to radiation than the adult flies which contain comparatively few dividing cells; it is for the same reason that X-rays are used in the treatment of cancer, the rapidly dividing cancer cells being much more sensitive to the radiation than the non-dividing surrounding tissues.

The genetic effects of ionising radiations have been studied in a wide variety of organisms and have led to several important conclusions. First, and perhaps most important, is that ionising radiation induces both gene mutations and chromosome breaks and that the frequency of each of these aberrations is directly proportional to the dose; thus in *Drosophila* sperm a dose of 2000 r (r, the roentgen unit, measures the number of ionisations occurring in a given volume of air and hence the amount of radiation) induces about 6 per cent sex-linked recessive lethal mutations, while a dose of 4000 r increases the frequency to about 12 per cent, and so on (figure 10.6). This linear relationship

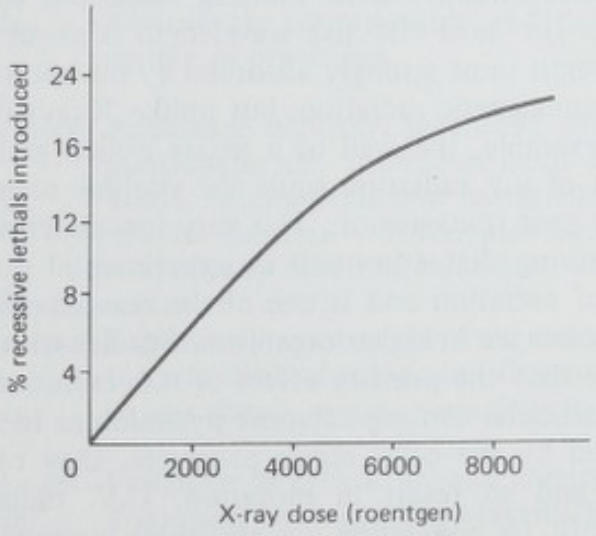


Figure 10.6 X-ray induction of mutation. The percentage of sex-linked recessive lethals induced by X-rays is directly proportional to the dose at low doses. At higher doses the response falls off because more than one lethal may be induced on a single X chromosome and the technique only measures the number of X chromosomes carrying at least one recessive lethal.

is found over a wide range of doses, but when sex-linked recessive lethals are induced by high doses of radiation their *apparent* frequency is lower than expected because more than one lethal mutation may be induced on the same chromosome; the Muller-5 test measures the frequency of X-chromosomes carrying *one or more* recessive lethals and not the actual frequency of recessive lethal mutations. Note also that whereas the frequency of chromosome *breaks* is proportional to the dose of iradiation the frequency of structural changes, such as inversions, translocations and deletions, is not; this is because these abnormalities require *two* breaks in the *same* cell followed by the rejoining of the fragments in a new combination so that a linear relationship is not expected.

The second important conclusion is that the effects of radiation are cumulative. In *Drosophila* sperm the number of mutations induced is proportional to the amount of irradiation received and is independent of the way in which the dose is administered; a low intensity of irradiation given for a long period of time (chronic irradiation), a high intensity given for a short period of time (acute irradiation) or two or more small doses given several hours apart (called dose-fractionation) all produce the same number of mutations. In other organisms this exact relationship does not hold. In mice spermatogonia, for example, chronic irradiation produces fewer mutations than the same dose of acute irradiation while dose-fractionation produces fewer mutations than an equally large single dose. However, it is true to say that even the smallest increase in the dose of irradiation received by a cell increases the probability of mutation occurring in that cell. Since most mutations that occur in natural populations are harmful rather than beneficial, unnecessary exposure to irradiation is to be avoided at all costs.

Ultraviolet Radiation

Although much less effective than ionising radiation, u.v. radiation can also induce mutations. The most effective wavelength is about 270 nm, corresponding to the wavelength most strongly absorbed by nucleic acids. Like X-rays, u.v. light is an electronmagnetic radiation, but unlike X-rays it is only very weakly penetrating—for example, the wall of a maize pollen grain allows only 30 per cent transmission of u.v. radiation while the vitelline membrane of a hen's egg allows only 8 per cent transmission. This very low penetration presents obvious difficulties in ensuring that every cell in experimental populations receives an equivalent dose of radiation and is one of the reasons why u.v. radiation is so seldom used as a mutagen in higher organisms. Studies with micro-organisms (see chapter 16) show that the primary effect of u.v. radiation is the photolysis of pyrimidines, in particular causing adjacent pyrimidines to form dimers; if these cannot be repaired by the cells' repair processes, they can cause errors at the next replication and so result in mutation. U.V. radiation can also cause mutations indirectly, by converting u.v.-absorbing compounds into radicals and peroxides, in turn capable of causing mutation.

Chemicals as Mutagens

In 1943, the first chemical mutagen, mustard gas, was discovered by Charlotte

Auerbach and J. M. Robson working with *Drosophila*, but as the work was classified it was not until after the war that their discovery became generally known. This was a scientific discovery of the utmost importance as it suggested that it might be possible to treat cancers with specific chemicals instead of by irradiation. Their discovery was not entirely fortuituous as knowing that mustard gas had much the same effect as X-rays in inhibiting cell division and causing inflammation led to a suspicion that it might also be mutagenic. It was soon found that mustard gas and nitrogen mustard, in addition to causing mutation in a wide range of organisms, also caused chromosome breaks and that the lesions caused by these radio-mimetic substances seemed indistinguishable from those produced by ionising radiations.

A wide range of substances, many of them known carcinogens, are now known to cause mutations in organisms as diverse as phage and mice. Many of these compounds are widely used in industrial processes while others are

Table 10.3 Some chemical mutagens and their effects

1. *Ethylenimine and derivatives*	
Representative uses	Textile industry (dying, crease- and water-proofing), agriculture (insecticides and soil conditioners), petroleum industry.
Harmful effects	Toxic and carcinogenic; induces mutations in a variety of organisms and chromosome aberrations in human cells.
2. *Thiotepa*	
Representative uses	Textile industry (dying, flame- and water-proofing).
Harmful effects	Induces mutations in mice and chromosome aberrations in human cells.
3. *Mustard gas*	
Representative uses	Applications in the polymer industry.
Harmful effects	Teratogenic, carcinogenic and mutagenic in a wide variety of organisms.
4. *Nitrosamines*	
Representative uses	Petroleum industry, nematode control, rocket fuels, plasticisers; also present in many foods and tobacco.
Harmful effects	Toxic, carcinogenic and mutagenic in *Drosophila* but not in bacteria—nitrosamines probably have to be enzymically modified to be active.
5. EDTA (*ethylenediaminetetraacetic acid*)	
Representative uses	Extensively used in the food industry for promoting the retention of colour and flavour.
Harmful effects	Causes chromosome aberrations in higher organisms.
6. *Captan*	
Representative uses	Fungicide in agriculture and horticulture.
Harmful effects	Toxic and teratogenic; mutagenic in *E. coli*.
7. *Streptonigrin*	
Representative uses	Antibiotic, cancer chemotherapy.
Harmful effects	Carcinogenic and mutagenic; induces chromosome aberrations in the cells of higher organisms.

commonly used as pesticides, insecticides, antibiotics and food additives (table 10.3). Thus there is a growing need to identify mutagenic chemicals and to ensure protection against them. Some of the known chemical mutagens are highly toxic (for example nitrogen mustard) and so can easily be detected before they can produce any significant genetic damage, but others are much more mutagenic than toxic and so could easily escape detection until serious genetic damage has been caused. Clearly we are most concerned about potential genetic damage in man, but since man cannot be used as an experimental organism we can only extrapolate from the results of mutation tests on other organisms; how justified we are in making these extrapolations remains to be seen, but at present they are usually the only guide lines we have. Thus a compound that is mutagenic in bacteria, *Drosophila*, or mice is regarded as a potential mutagen in man (see page 303); it does seem probable that most potential mutagens will turn out to be actual mutagens in man, provided that they can get into the germ cells.

The Molecular Basis of Mutation

Once the Watson–Crick model for the structure and replication of DNA was firmly established, it seemed clear that gene mutations acted by altering the sequence of nucleotides along the DNA molecule so that the functioning of the gene and, ultimately, the phenotype of the organism was affected. We now recognise that gene mutation can change the informational content of a gene in at least two ways—first, by a base substitution changing one base pair to a different base pair (or in a single-stranded nucleic acid by changing one base for another) and, second, by adding or deleting one or more base pairs. We have already outlined the effect on protein structure when these mutations occur in a structural gene, and we will now consider the sequences of events that can give rise to gene mutation when nucleic acids are exposed to a particular chemical mutagen. Although many compounds are known to be mutagenic, mainly as a result of testing them in microbial systems, only in certain instances is the mechanism of their action reasonably well understood, and it is with these that we are primarily concerned. These compounds can be considered under three headings, the base analogues, compounds that cause chemical alterations to the nucleic acids, and compounds that bind to DNA.

Base-Analogue-Induced Mutation

An analogue is a compound whose molecular structure is very similar to that of a naturally occurring compound and so may be able to substitute for it in some important biological reaction. The base analogues, first investigated in detail by Ernst Freese, are important because a few of them can replace one or other of the natural bases during the replication of DNA, cause errors of pairing and so the substitution of one base pair for another. The first analogue to be investigated was 5-bromouracil (BU), an analogue of thymine and now one of

the best known and most extensively studied compounds which cause point mutation; BU and its related analogues 5-chlorouracil and 5-iodouracil are known to cause mutation in phage and bacteria. If *E. coli* is grown in the presence of BU the analogue becomes incorporated into DNA in place of

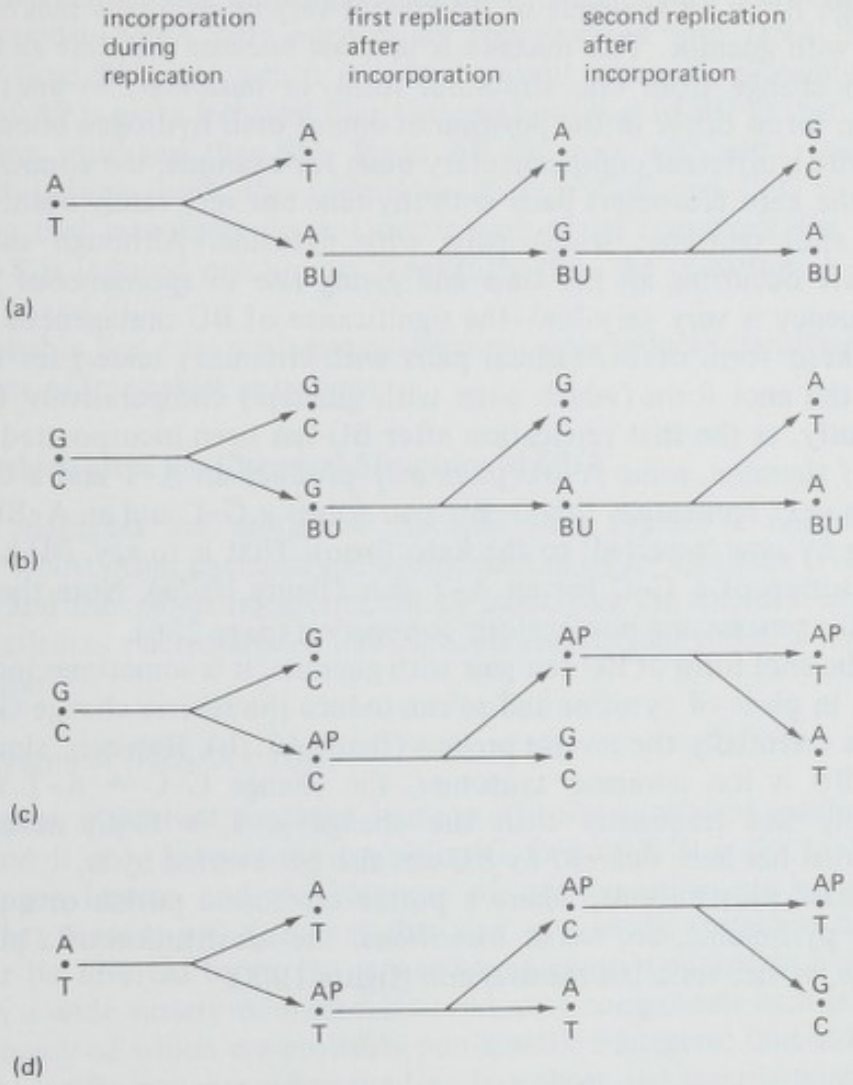


Figure 10.7 Base analogue-induced mutation.

(a) Induction of the transition A–T → G–C by 5-bromouracil.

(b) Induction of the G–C → A–T by 5-bromouracil.

(c) Induction of the transition G–C → A–T by 2-aminopurine.

(d) Induction of the transition A–T → G–C by 2-aminopurine.

(a) and (c) During replication the common tautomer of the analogue is incorporated into DNA instead of one of the natural bases, and at the first replication after incorporation the analogue may pair with a different natural base leading to a base-pair substitution at the next replication (error of replication).

(b) and (d) During replication there is an error of pairing and the rare tautomer of an analogue is incorporated into DNA. At the first replication after incorporation the analogue pairs normally, leading to a base-pair substitution at the next replication (error of incorporation). Note that errors of replication are more common than errors of incorporation so that while BU induces the transition A–T→ G–C more frequently than the reverse transition G–C→ A–T, AP induces the change G–C→A–T more frequently than the change A–T → G–C.

thymine and, either by growing the cells in the absence of free thymine or by using the corresponding deoxyribonucleotide (5-bromodeoxyuridine, BUdR), it is possible to increase the amount of analogue incorporated until almost all the thymine has been replaced.

Although BU is an analogue of thymine it very occasionally makes a mistake and pairs with guanine. This mistake is possible because BU, like all the natural bases, can change from one structural form, or *tautomer,* to another. These tautomeric forms differ in the position of one of their hydrogen bonds and each can pair with a different complementary base; for example, the common form of adenine (the keto tautomer) pairs with thymine but very rarely a shift occurs to the rare enol tautomer which pairs with cytosine. Although such pairing mistakes are occurring all the time and giving rise to spontaneous mutations, their frequency is very very low—the significance of BU mutagenesis is that the common keto form of BU (which pairs with thymine) undergoes tautomeric shifts to the enol form (which pairs with guanine) comparatively frequently. Consequently, at the first replication after BU has been incorporated into DNA in place of thymine, some A-BU pairs may produce an A-T and a G-BU pair, and at the next replication this G-BU pair forms a G-C and an A-BU pair (the BU having by now 'reverted' to the keto form). That is to say, BU has induced the substitution of a G-C for an A-T pair (figure 10.7a). Note the similarity between this process and post-meiotic segregation (page 286).

Since the enol form of BU can pair with guanine, it is sometimes incorporated into DNA in place of cytosine and so can induce the reverse change G-C → A-T by what is essentially the reverse process (figure 10.7b). However, since the enol form of BU is less common tautomer, the change G-C → A-T is induced considerably less frequently than the change A-T → G-C; nevertheless, a mutation that has been *induced* by BU can also be reverted by it.

These base substitutions, where a purine replaces a purine or a pyrimidine replaces a pyrimidine, are called *transitions;* the substitution of a purine for a pyrimidine, or vice versa is a *transversion* (figure 10.8).

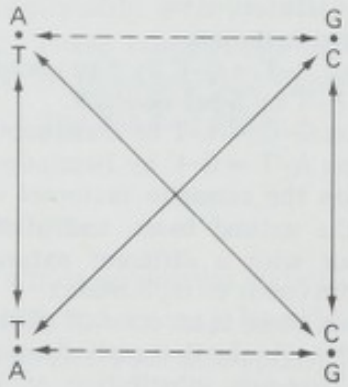


Figure 10.8 Transitions and transversions. Base-pair substitutions are transitions when a purine replaces a purine or a pyrimidine replaces a pyrimidine (dotted lines) and transversions when a purine is replaced by a pyrimidine and vice versa (solid lines).

We must remember that induced mutations are still extremely rare events that can usually be detected only by screening millions of organisms.

Another analogue of thymine, 2-aminopurine (AP), is also mutagenic in certain organisms, including T4 and *E. coli*, and whether it is mutagenic probably depends on the cell being able to convert it into the corresponding deoxynucleotide. AP is only very rarely incorporated into DNA, much less frequently than BU, and yet it is a very potent mutagen; probably the rare tautomer of AP is more frequent than the rare tautomer of BU so that AP makes more pairing mistakes than BU. Since AP can pair with either thymine or cytosine, it induces both the transitions A-T → C-C and G-C → A-T, but because the two tautomers do not occur with equal frequency the transition G-C → A-T is induced much more frequently than the transition A-T → G-C (figure 10.7c and d).

It is probable that base analogue mutagenesis only induces transitions and not transversions or frameshift mutations.

Mutagens which alter the Chemical Structure of DNA

The base analogues are mutagenic because they replace a normal base in *replicating* DNA; other chemical mutagens, such as hydroxylamine (HA), nitrous acid (NA) and the alkylating agents, act by modifying the chemical composition of the constituent nucleotides of nucleic acids and so their action is independent of replication.

(i) Hydroxylamine (NH_2OH; HA)

This is a most important mutagen because its only significant reaction is with cytosine and it only induces the transition G-C → A-T. It is the most specific point mutagen known and the response of any particular site within a gene indicates that there is a G-C pair at that site; as a result, mutations induced by HA *cannot* be reverted by it. HA is a reducing compound and in living cells it reacts with a wide variety of molecules producing compounds such as hydrogen peroxide, many of which are probably non-specific mutagens; thus although HA is a highly specific mutagen when used on free phage and transforming DNA, its specificity may be lost when it is used to treat an intact cell, such as a bacterium. It is thought that HA acts by changing cytosine into a base which pairs with adenine instead of with guanine (figure 10.9).

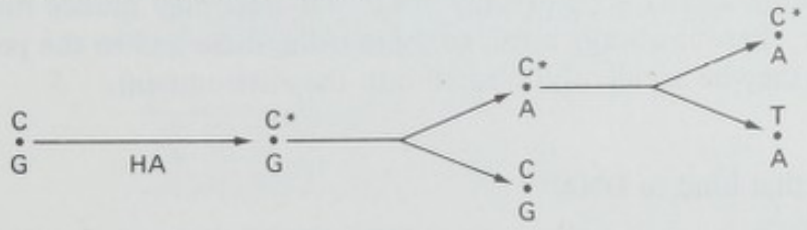


Figure 10.9 One way transition G–C → A–T induced by hydroxylamine. Hydroxylamine converts cytosine into a modified base (C*), which can pair with either guanine or adenine. This modification occurs independently of replication and ultimately leads to a G–C → A–T transition.

(ii) Nitrous Acid (HNO_2; NA)

This is a very potent mutagen known to induce mutations in a wide variety of organisms including TMV, T2, T4, *E. coli* and *Neurospora.* In both TMV and T4 nitrous acid induces transitions in both directions (A–T↔G–C) and NA-induced T4*r*II mutants are, as is expected if they are transitions, revertible by either nitrous acid or by base analogues. The principal action of nitrous acid is the oxidative deamination of guanine transforming it into xanthine, but since xanthine cannot pair with any other base this change may be lethal to the cell. Less frequently NA changes cytosine into uracil and adenine into hypoxanthine; since hypoxanthine can pair with cytosine and uracil can pair with adenine this leads to the transitions A-T → G-C and G-C → A-T in the same way as the base analogue substitutions. Nitrous acid is also known to produce deletions in T4 and *E. coli* and frameshift mutations in yeast.

(iii) The alkylating agents

Many different alkylating agents are mutagenic in a wide variety of organisms including phage, bacteria, *Vicia, Neurospora* and *Drosophila.* Among them is mustard gas, the first chemical mutagen to be discovered. Although the mutational *specificity* of some of these compounds is well known the precise *mechanism* of their action is not. Three alkylating agents very commonly used as mutagens are ethylethane sulfonate (EES), ethylmethane sulphonate (EMS) and N-methyl-N-nitro-N-nitrosoguanidine (NG), and each has a complex spectrum of action. EES and EMS (1) will strongly induce the reversion of HA-induced T4*r*II mutants and so *strongly* promote G−C→A−T transitions; (2) much less effectively, will revert mutants that are both induced *and* reverted by the base analogues but *not* by HA—these mutations must be A−T→G−C transitions; (3) rarely will revert mutants that are *not* reverted by base analogues *nor* by acridines (which induce frameshift mutations)—these reversions are presumed to be due to G−C→C−G or G−C→T−A *transversions*; (4) may also induce frameshift mutations. NG is also an extremely potent mutagen in micro-organisms and it induces transitions, transversions and large deletions, but probably not frameshift mutations.

Alkylating agents are important because they are very extensively used in industry and so are a potential danger as pollutants of our environment. Since many alkylating agents are not only toxic but also may induce mutation and cause cancer, there is always a risk to those using them and to the population at large should they be accidently released into the environment.

Compounds that bind to DNA

The acridines are important because they were the first compounds found which could induce frameshift mutations in phage during their intracellular growth. One of the acridines, proflavin, was the mutagen used by Crick to induce *r*II mutants in phage T4 (chapter 12). The most important characteristic of these

proflavin-induced mutants is that they can be reverted by proflavin but *not* by the base analogues. This is because the acridines induce frameshift mutations due to the addition (+) or deletion (–) of one or more base pairs—thus an *r*II mutant induced by the addition of a base pair can sometimes be reverted by the deletion of an adjacent base pair, so restoring the phase of the code, but *not* by a base pair substitution.

Although the mutagenic effect of the acridines is confined to phages, large numbers of frameshift mutations can be induced in bacteria by using the structurally related ICR compounds—these are acridines with an attached side chain, sometimes an alkylating agent.

The acridines (for example, proflavin, acriflavin and acridine yellow) and the ICR compounds are rather flat molecules that are able to bind to DNA by sliding in between (intercalating) adjacent base pairs forcing them apart. There is now evidence that the mutagenic action of the acridines on phage is correlated with genetic recombination, and it is believed that the intercalated acridine molecule distorts the double helix of DNA and leads to misalignment during genetic recombination; as a result, unequal crossing-over occurs and produces two recombinant molecules, one with too many and the other with too few base pairs (figure 10.10). We do not know whether the ICR compounds cause mutation in bacteria by a similar mechanism; an alternative possibility is that the distortions in the DNA molecule cause the addition or deletion of a base pair during replication.

Frameshift mutations have been positively identified in phage, bacteria and yeast, but not in *Neurospora* or in any higher organism, although certain mutants with unusual properties have been interpreted as frameshifts.

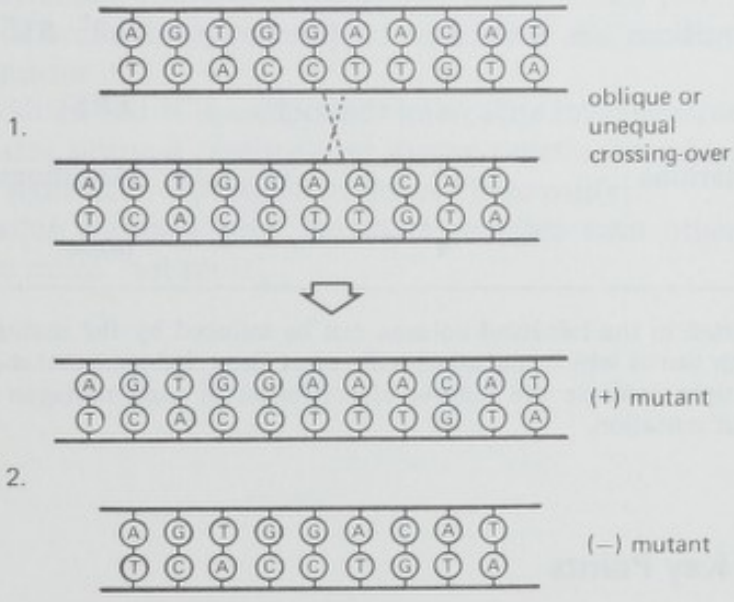


Figure 10.10 An explanation of acridine-induced mutagenesis. It is thought that the intercalation of an acridine molecule between two adjacent base pairs distorts the molecule so that there is misalignment during genetic recombination. Crossing-over can now occur between non-homologous base pairs (1) producing recombinant molecules with too many or too few base pairs (2); this is called oblique or unequal crossing-over.

Mutation is not a completely Random Process

Although we are accustomed to thinking of mutation as a random process, this is not quite true. If many allelic point mutants are mapped by fine structure analysis the resulting mutational spectrum identifies many different sites (that is, base pairs) within the gene and records the number of times mutation has recurred at each site. In the T4 *r*II region mapped by Benzer, 2400 spontaneous mutants were mapped at 308 different sites (page 137) but each site did not mutate at the same frequency. At many sites mutation to *r*II occurred only once or twice, many mutations occurred up to twelve times, one exceptional mutation occurred 517 times (*r*17) and another 298 times (*r*131). These highly mutable sites are called 'hot spots'. The mutational spectrum for 5-bromouracil-induced mutations also shows hot spots, but what is of interest is that these are in different positions to the hot spots for spontaneous mutation.

Since there are effectively only two different base pairs (A-T and C-G), a highly mutable hot spot must have not only the corect base pair at that site but also the correct sequence of adjacent base pairs; it would appear that a mutagen 'recognises' a particular base sequence more often than other sequences, and that each mutagen 'recognises' a different sequence (see page 304)

Table 10.4 Mutational spectra

Mutational change	Induced by						Can be reverted by
	BU	AP	NA	HA	EES	ACR	
G–C → A–T transitions	++	+	++	++	++		AP^{++} BU^{+} (A–T → G–C)
A–T → G–C transitions	+	++	++		+		AP^{+} BU^{++} HA^{++} (G–C → A–
Transversions					+		EES?
Frameshift mutations			+		+	++	Acridines only
Deletions			+				none

The mutations listed in the left-hand column can be induced by the mutagens shown. The right-hand column shows which mutagens will revert these induced mutations back to wild type. The plus signs indicate the relative effectiveness of each mutagen in inducing the different classes of mutation.

Summary of Key Points

(1) Mutation is the ultimate source of all *new* genetic variation, and without it evolution could not occur.

(2) Mutation takes place at the level of either (i) the chromosomes, by increasing the number of chromosomes or by rearranging the genetic material, or

(ii) the constituent nucleotides, by changing one base pair to another or by adding or deleting one or more base pairs.

(3) Although most (but not all) mutations in higher organisms are recognised because they alter the appearance of individuals carrying them, most mutations in micro-organisms result in specific biochemical defects.

(4) Mutation rates, expressed as the number of mutations per cell (or per individual) per cell division (or per generation) can be estimated by a number of methods; they are most easily measured in bacteria where selective methods can be used to detect very rare mutations.

(5) Every wild-type allele has a characteristic rate of forward mutation and every mutant allele a characteristic rate of back mutation.

(6) The rate of mutation can be increased by exposure to a mutagen.

(7) Radiations, particularly ionising radiations, are powerful mutagens and can cause genetic damage by (i) causing chromosome breaks by fracturing the sugar-phosphate backbone of DNA molecules, and (ii) causing changes in the DNA which lead to anomalies when the chromosomes subsequently replicate.

(8) The frequency of point mutations and of chromosome breaks is directly proportional to the dose of radiation; the genetic effects of radiation are cumulative.

(9) Many chemicals are mutagens, but for the most part their mode of action is unknown.

(10) Base analogues cause base-pair substitutions by replacing one of the naturally occurring bases during DNA replication; at the next replication an error of pairing occurs and eventually leads to a base-pair substitution. These analogues induce the transitions A-T → G-C and G-C → A-T.

(11) Hydroxylamine converts cytosine into a base which pairs with guanine and adenine—this occurs independently of replication and results in the specific G-C → A-T transition.

(12) The acridines and ICR compounds intercalate into the structure of DNA and probably cause unequal crossing-over during genetic recombination, leading to recombinant molecules with added or deleted base pair(s).

(13) Some sites within a gene are more mutable than others; extremely mutable sites are called 'hot spots'.

11 Genes and Proteins

Be wise today; 'tis madness to defer.
Edward Young (1863-1765)

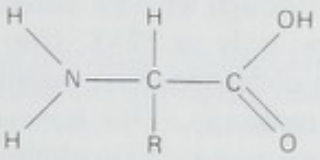
In previous chapters we have considered the structure, replication and transmission of the genetic material, and we will now explore the fields of biochemical genetics, in particular examining the relationships between gene structure and protein structure and seeing how almost every metabolic process in the living cell is ultimately under the direct control of the genes.

From the point of view of both structure and function the proteins are the most important and the most widely occurring constituent of living cells. Even in a comparatively simple organism like *E. coli* there are probably about 2000 different types of protein, making up one-half of the dry weight of the cell, while in humans it is estimated that there are 100 000 types of protein constituting 70 per cent of their dry weight. Although there may be very close similarities between the corresponding proteins in different organisms they may not be identical; every species makes some unique proteins, and even between two individuals of the same species there may be small differences in what appears to be the same protein. Many proteins play largely structural roles; thus the protein coat of a virus particle stabilises and protects the nucleic acid, collagen is a fibrous protein, which is an important constituent of bone, tendons and ligaments, myosin is the main muscle protein, while keratin is found in hair, wool and horns. Other proteins are essential to build up and to break down large molecules in the cell, so controlling its metabolism. These *enzymes* act as biological catalysts by promoting and speeding up chemical changes without becoming changed themselves, so that a single molecule of enzyme can catalyse the synthesis or breakdown of many hundreds of molecules of substrate. Enzymes have one or more *active sites* which specifically bind the molecules of substrate so as to bring their reactive groups closer together, so forming an enzyme-substrate complex. After covalent bonds have formed between the substrate molecules, the products are released and the enzyme molecule is free to catalyse a further reaction (this concept of enzyme action is represented diagrammatically in figure 14.8). These enzymes are highly specific and each will usually catalyse only a single type of reaction.

The Structure of Proteins

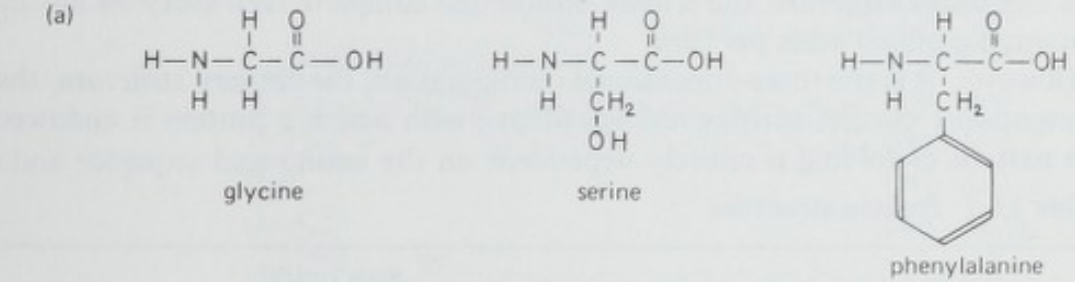
Proteins are complex macromolecules made up of one of more *polypeptide chains.* These polypeptides, like nucleic acids, are linear aggregates of building blocks and the difference between molecules with different specificities lies in

the orders of the subunits along the chains and in the lengths of the chains. The building blocks of polypeptides are the twenty common α-amino acids, a group of related compounds characterised by having a -COOH carboxyl group (often negatively charged) and an $-NH_2$ amino group (often positively charged) attached to the same carbon atom (except proline, which has no amino group). They have the general formula



where R (for radical) represents a specific side group. The formulae for some of the twenty 'standard' amino acids are shown in figure 11.1.

The amino acids can be enzymatically joined together by the carboxyl group of one amino acid covalently bonding to the amino group of another, forming a peptide bond and releasing a molecule of water. Very many amino acids can successively join in this way forming a long chain of amino acids, a polypeptide chain (figure 11.1b), with one free carboxyl and one free amino group. Although



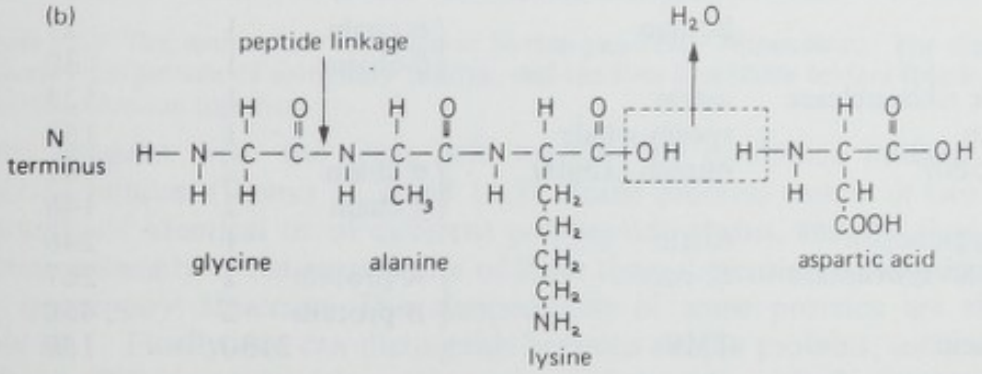


Figure 11.1 Amino acids and polypeptides.

(a) Three of the twenty acids found in proteins–they differ only with respect to the side chain attached to the alpha carbon.

(b) A polypeptide chain. An aspartic acid residue is to be added on to the C terminal, releasing a molecule of water.

any two amino acids joined by a peptide bond are, strictly speaking, a protein molecule, most cellular proteins are complex macromolecules made up of one or more polypeptide chains folded into a highly ordered three-dimensional structure. The activity of a protein is totally dependent on the spatial arrangement of the amino acids within the three-dimensional structure, and just how the polypeptide chain folds into this configuration is, in turn, dependent on the precise sequence of the amino-acid residues along it, the *primary structure* of the protein macromolecule. Although we now know the amino-acid sequences of many different proteins, it was only in 1953, after ten years of intensive work that Frederick Sanger and his colleagues first established the sequence of a very simple protein, the insulin molecule. This hormone contains only fifty-one amino acids and consists of two polypeptide chains held together by disulphide bridges (figure 11.2); its structure is of unusual interest because the immediate gene product is a single polypeptide eighty-four amino acids long, thirty-three amino acids subsequently being enzymatically removed from its centre. The techniques pioneered by Sanger paved the way for the determination of the primary structures of much larger polypeptides such as pancreatic ribonuclease (figure 11.3) and tryptophan synthetase-A protein, one of the largest polypeptides sequenced.

Large sections of the linear polypeptide usually assume a helical shape brought about by hydrogen bonding between nearby groups on the same chain. This *secondary structure*, the α helix, makes one complete turn every 54 nm and contains 3.6 amino acids per turn.

However, it is the three-dimensional configuration, the *tertiary structure*, that is responsible for the activity and specificity with which a protein is endowed. The pattern of folding is entirely dependent on the amino-acid sequence and is

Table 11.1 Protein structure

Protein	Source	Sub units/ molecular complex		Amino acids/ subunit
Insulin*	human	α chain	1	21
		β chain	1	30
Pancreatic ribonuclease	cattle		1	124
Myoglobin	sperm whale		1	153
Haemoglobin	human (adult)	α chain	2	141
		β chain	2	146
Chymotrypsinogen	cattle		1	246
Tryptophan synthetase	*E. coli*	A protein	2	267
		B protein	2	*c.* 450
Coat protein	TMV		2130	158

* Initially produced as a single polypeptide chain.

This table gives the numbers of sub-units per molecular complex and the numbers of amino acids per sub-unit of some representative proteins. An 'average' polypeptide chain is about 250 amino acid residues long.

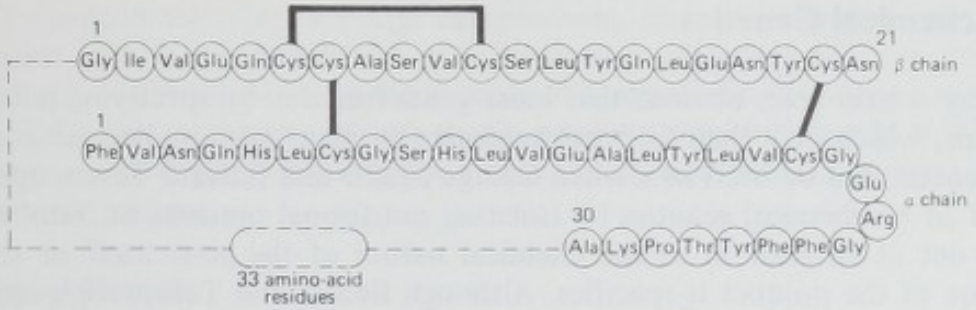


Figure 11.2 The primary structure of insulin. The circles represent the fifty-one amino-acid residues found in the α and β chains of beef insulin, the heavy lines indicate disulphide bridges between adjacent cysteine residues. The α and β chains are initially synthesised as one long polypeptide eighty-four residues long, known as proinsulin. Thirty-three residues are subsequently cleaved from the centre of this polypeptide to produce the two peptide fragments, which are the α and β chains of the insulin molecule. The amino-acid sequence of the insulin molecular complex was determined by F. Sanger.

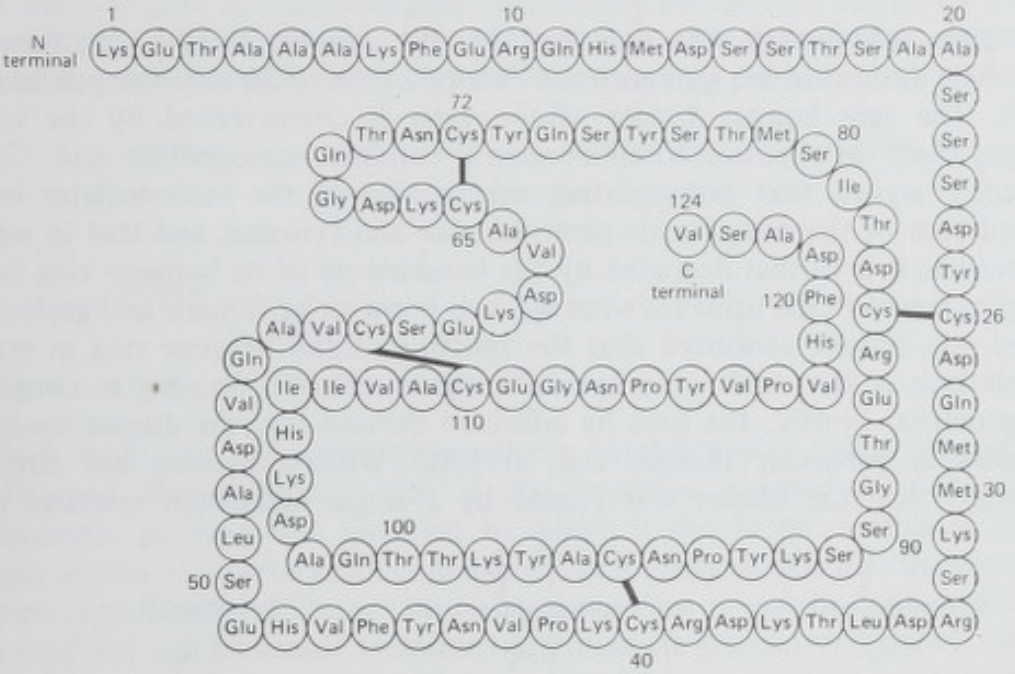


Figure 11.3 The amino-acid sequence of bovine pancreatic ribonuclease. The diagram also indicates the pattern of secondary folding, and the four disulphide bridges (black bars) that hold the structure together.

often irregular with the folds held together by disulphide bridges between cysteine residues (figures 11.2 and 11.3). Some proteins consist of two or more sub-units of identical or of different polypeptide chains, and the final stage in protein assembly is the aggregation of these three-dimensional subunits to form the *quaternary structure*. The compositions of some proteins are shown in table 11.1. Finally, we can distinguish between simple proteins, such as insulin and the TMV coat protein, and *conjugated* proteins, which contain another component referred to as a prosthetic group. Examples of conjugated proteins are myoglobin and haemoglobin, which transport oxygen and other gases in animals, while many enzymes are proteins conjugated with metal-containing pigments or vitamins.

Biochemical Genetics

Today it may seem obvious that most genes function by specifying polypeptide chains, which may then fold and aggregate to form macromolecular complexes of protein, but before 1941, when George Beadle and Edward Tatum opened the field of biochemical genetics by isolating nutritional mutants of *Neurospora*, it was not at all clear as to the chemical nature of the gene itself or the exact nature of the product it specifies. Although Beadle and Tatum were the first to establish a positive correlation between a particular gene and a particular enzyme, so leading to the formulation of their *one gene-one enzyme* hypothesis, the idea of a relationship between genes and enzymes was already long established and had been slowly developing over the previous thirty years.

Inborn Errors of Metabolism

The first relationship was described by the famous English physician Sir Archibald Garrod in his famous book *Inborn Errors of Metabolism* published in 1909. The rare human disease *alkaptonuria* is characterised by the urine's turning black on exposure to air because it contains homogentisic acid. Garrod correctly argued that homogentisic acid is one of the intermediates in the degradation of the amino acids phenylalanine and tyrosine, and that in normal individuals it is further degraded by the breaking up of its benzene ring before being excreted in the urine (as what we now know to be fumaric and acetoacetic acids). He further conceived that the splitting of the benzene ring in normal metabolism is the work of a special enzyme which is wanting in congenital alkaptonuria. Finally, the data he collected showed that the disease tended to re-occur in particular families and, in 1902, William Bateson had correctly surmised that the disease was caused by a single Mendelian recessive gene.

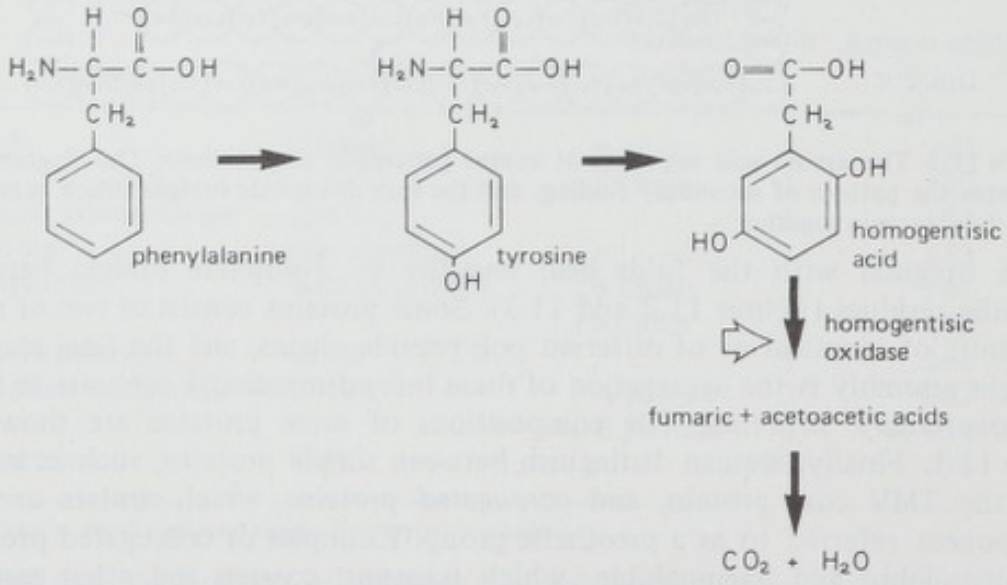
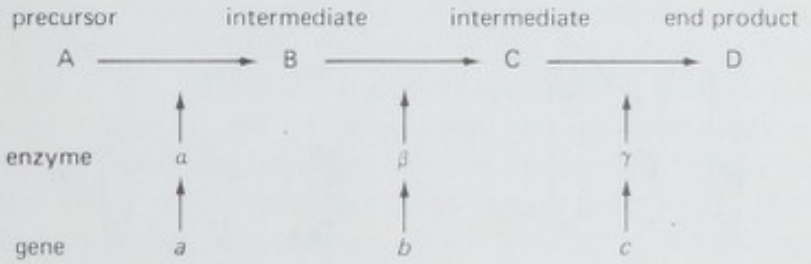


Figure 11.4 The degradation of phenylalanine and tyrosine in man. The open arrow indicates the block in alkaptonuria.

Garrod's fundamental idea was that alkaptonuria, and other 'inborn errors of metabolism', could be interpreted as a block at a particular point in normal intermediary metabolism due to the absence of a genetically determined enzyme (figure 11.4). It is interesting to note that it was not until 1958 that B. N. La Du was able to confirm the absence of the enzyme, homogentisic acid oxidase, in an alkaptonuriac patient.

Biochemical Mutants

More detailed knowledge of the relationships between genes and enzymes stems from the work of Beadle and Tatum on the bread mould *Neurospora crassa*. A wild-type strain of *Neurospora* can grow on a minimal medium (MM) containing inorganic salts, a sugar and the vitamin biotin. From these simple compounds *Neurospora* can synthesise all the nucleotides, amino acids, vitamins and other molecules that are required for growth and reproduction. In living cells the synthesis of these complex biochemical compounds occurs by a series of small steps each catalysed by its own enzyme, while the product of each step is passed to the next enzyme so that the following step can take place



Each such series of reactions is called a *biochemical* or *metabolic pathway*. Beadle and Tatum argued that if the enzymes involved in a particular biosynthetic pathway are specified by genes, it should be possible to obtain mutant strains that have a defect in one of these genes so that the corresponding enzyme is no longer produced; thus a strain mutant in gene *b* (above) will lack enzyme β and will be unable to convert intermediate B to intermediate C, and no end product will be produced. If D is an essential compound (an amino acid, for example), the mutant strain will no longer grow on minimal medium unless it is *supplemented* with substance D. In effect, Beadle and Tatum hoped to study how genes work by causing defects in them and then tracing back to see the nature of the defect.

They *induced* defective or mutant strains by exposing asexual spores to X-rays or ultraviolet light so as to speed up the process of gene mutation. After a rather complicated procedure to ensure the purity of the treated strains, each strain was grown on complete or nutrient medium (CM) and then subcultured on to minimal medium (figure 11.5). Those strains which failed to grow on minimal medium were retained as being defective in a function essential on minimal medium. The biochemical nature of the defects were determined by subculturing from complete medium on to minimal medium supplemented with either the amino acids or the vitamins or the nucleotides; if, for example, the strain only

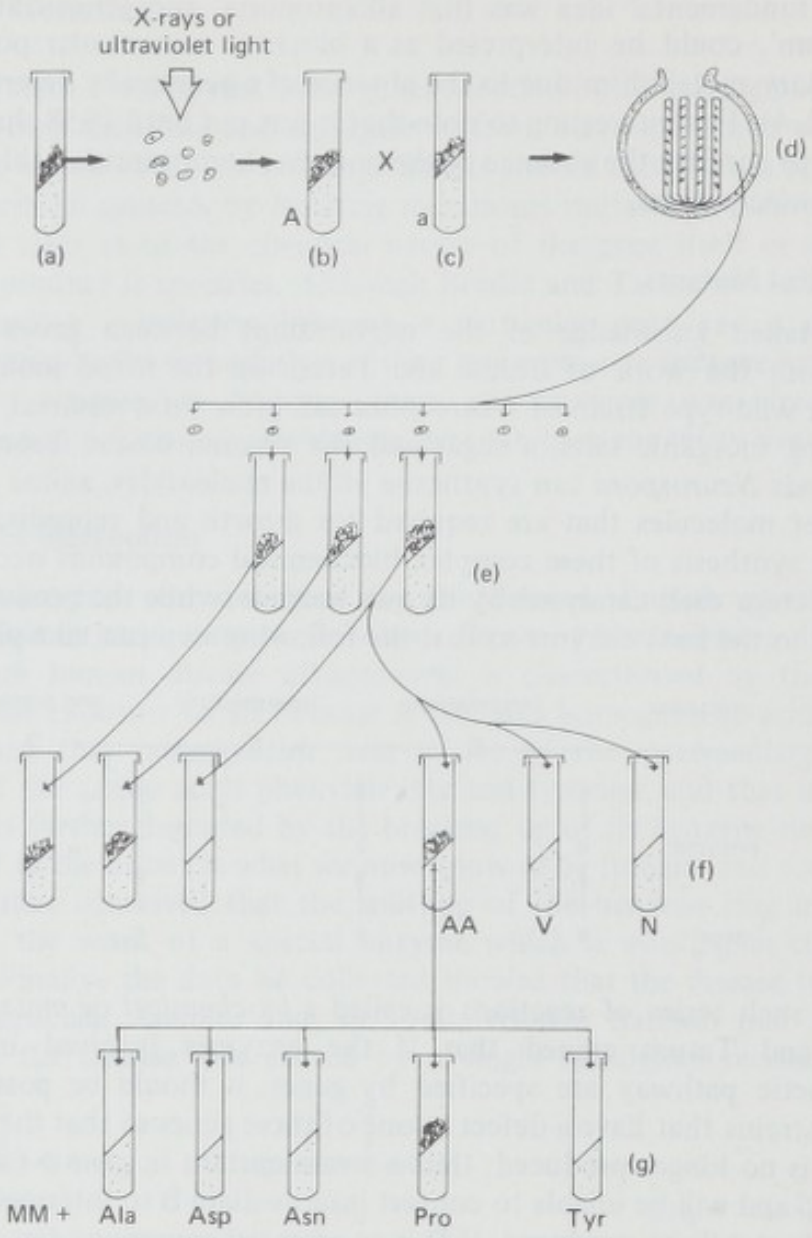


Figure 11.5 The method used by Beadle and Tatum to isolate biochemical mutants of *Neurospora crassa*. The isolation of a proline-requiring mutant is illustrated.

(a) Conidia are taken from a wild-type strain and mutagenised.

(b) The conidia are grown on complete medium.

(c) Each culture is crossed with a wild-type strain of the opposite mating type.

(d) Fruiting bodies with asci are formed.

(e) The ascospores are removed and grown individually on complete medium.

(f) Each culture that does not grow when transferred to minimal medium (left) is retained as a biochemical mutant; these are further tested (right) by subculturing onto minimal medium supplemented with either the amino acids (AA) or vitamins (V) or nucleotides (N).

(g) The mutant shown only grows on minimal medium supplemented with the amino acids. The particular amino acid required for growth (proline) is identified by subculturing onto minimal medium supplemented with the individual amino acids.

grew on minimal medium supplemented with the amino acids, it was further tested by subculturing on to a series of minimal-medium tubes each containing a single amino acid supplement. In figure 11.5 the mutant strain has only grown on minimal medium supplemented with arginine, showing that this mutant is defective in one of the reactions in the arginine biosynthetic pathway. Finally, they crossed each mutant strain with the wild type so as to show that the mutant phenotype had resulted from an alteration within a single gene (chapter 6). By this method Beadle and Tatum examined over 68 000 spores and isolated several hundred genetically different mutants. As a consequence of their results they realised that the function of a gene could be equated with the presence of a specific protein and so they proposed their one gene-one enzyme hypothesis.

The One Gene-One Enzyme Hypothesis

This hypothesis states that the primary function of a gene is to direct the formation of one specific enzyme so promoting and controlling one particular biochemical reaction. Two important facts should be noted. Firstly, their results did not offer any evidence *against* an alternative suggestion that a gene might control the production of many enzymes. If this were so there would be a high probability that at least one of the enzymes specified by a particular gene would be vital to the organism and required even on complete medium; mutations within such genes would be lethal to the cell and would remain undetected. It was not until 1961 that experimentation largely discounted this hypothesis. Secondly, the hypothesis says nothing about the way in which a gene might be able to direct the formation of an enzyme.

The One Gene-One Polypeptide Hypothesis

Above all, more recent research has brought to light several facts which have necessitated modification of the Beadle and Tatum hypothesis. We know that many genes direct the synthesis of proteins which are not enzymes (for example, haemoglobin and insulin), so that it would be more accurate to say one gene-one protein. But even this is not adequate, as many proteins are complexes of two or more polypeptide chains (table 11.1) each of which is made separately in the cell and is specified by a different gene. A more correct restatement of the hypothesis is one gene-one polypeptide chain. Finally, we must remember that not every gene specifies a polypeptide; ribosomal RNA and the many different molecules of transfer RNA are all the direct products of gene action, but unlike messenger RNA they do not act as templates for protein synthesis.

With the realisation that each step in a biochemical pathway is controlled by a different gene it became possible to use mutant strains to work out the sequence of reactions in a biochemical pathway. The first genetic analysis of a biochemical pathway was made in 1944 by Adrian Srb and Norman Horowitz using arginine-requiring mutants of *Neurospora crassa*. They obtained fifteen mutants, all of which were unable to synthesise arginine; when these mutants were crossed with the wild type, both arginine-requiring and wild-type progeny

were recovered in equal numbers, showing that each strain differed from the wild type by a single gene mutation. When pairs of mutants were crossed together, some gave only arginine-requiring progeny and these mutants appeared to be due to separate, or independent, mutations within the same gene; other pairs gave many wild-type progeny and so were considered to be due to mutation within different genes. Altogether they identified seven genes involved in arginine biosynthesis. They were able to confirm which pairs of mutants were allelic and which were not by using the heterocaryon test.

The Heterocaryon Test

If two strains of *Neurospora* of the *same* mating type are grown together the hyphae can fuse and form a heterocaryotic mycelium, containing a mixture of the two genetically different types of nuclei in the same cytoplasm. This is yet another way that two mutants can be tested to see if they complement (see pages 113 and 137. If a heterocaryon is made from two non-allelic arginine-requiring mutants (that is, the mutations are in different genes), then both wild-type gene products will be present in the cytoplasm and the heterocaryon will grow (figure 11.6a); however, if the heterocaryon is made from two allelic mutants (that is, the same gene is mutant in both strains) it will not be able to grow because neither nucleus can provide the wild-type gene product (figure 11.6b). If the heterocaryon does grow then the mutations are probably non-allelic and in different genes (but see page 202 for exceptions to this generalisation).

The mutants were then tested to see how they responded to the chemically related compounds, ornithine and citrulline, both suspected as being precursors of arginine. Four of the seven classes (those due to mutations in the genes that

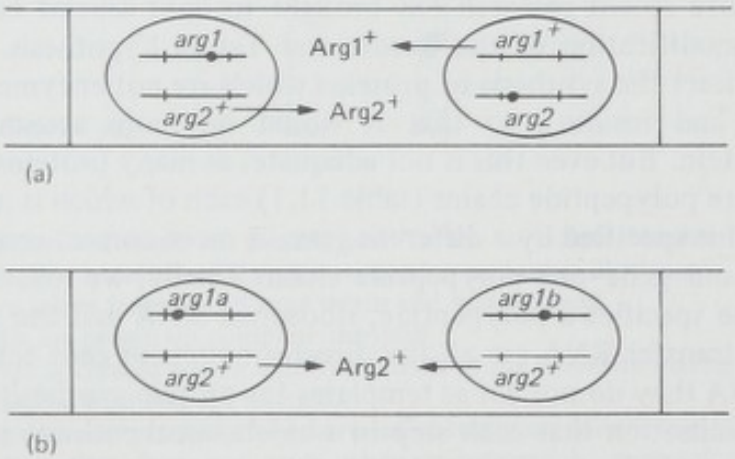


Figure 11.6 Complementation in a heterocaryon of *Neurospora*.

(a) A heterocaryon made from two arginine-requiring strains mutant in different genes. Both wild-type gene products are present in the cytoplasm and growth occurs; this is complementation.

(b) When both arginine-requiring strains are mutant in the *arg1* gene no *arg1*$^+$ gene product is produced and the heterocaryon cannot grow.

we now know as *arg4*, *arg5*, *arg6* and *arg7*) grew on minimal medium supplemented with *either* ornithine or citrulline, two classes (mutant in the *arg2* and *arg3* genes) grew *only* on citrulline and not on ornithine, and one class (mutant in the *arg1* gene) would grow on *neither* ornithine nor citrulline. All the mutants would grow on medium supplemented with arginine, and there were none which grew on ornithine but not on citrulline. Since it was highly unlikely that a mutant could grow when it is provided with an intermediate that occurs *before* the genetic block and not on one occurring *after* the block the biosynthetic pathway for arginine must proceed

→ → → → ornithine → → citrulline → arginine

arg4 *arg2* *arg1*
arg5 *arg3*
arg6
arg7

Apart from biochemical and genetic analysis there is a very simple method which enables us to determine whether a block in one mutant strain is before or after a block in another mutant. If we grow *arg1* on minimal medium it will be able to synthesise citrulline, but since this cannot be converted to arginine it will accumulate in the cell. In the same way *arg2* and *arg3* mutants will accumulate ornithine because it cannot be converted to citrulline. If an *arg1* and an *arg2* mutant are grown side by side on minimal medium containing just enough arginine to allow the commencement of growth, the citrulline accumulated by the *arg1* mutant will diffuse into the medium where it can be utilised by the *arg2* mutant; *arg2* will grow when citrulline is provided and although there will not be a lot of growth there is enough to be detectable (figure 11.7). The *arg1* mutant is said to *feed arg2*. Note that the block in the mutant that is fed (*arg2*) is always before the block in the feeding mutant (*arg1*).

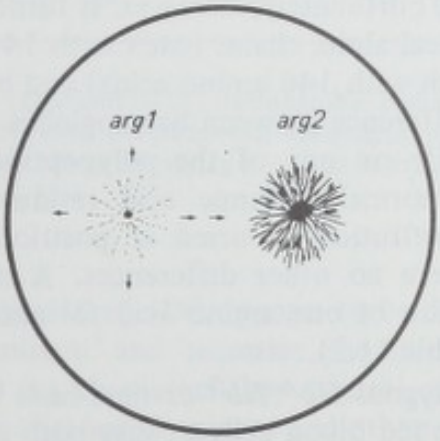


Figure 11.7 Feeding. The slight growth of the *arg1* mutant of *Neurospora* allows the accumulation of citrulline. This diffuses into the medium (arrows) and permits the growth of the *arg2* mutant, which will grow on citrulline or arginine but not on ornithine.

It was by this type of combined genetic and biochemical analysis that many of the cellular biosynthetic pathways were first elucidated.

While these experiments clearly demonstrate a close relationship between gene mutation and the presence or absence of a particular enzyme, they do not show what we have so far assumed, that the genes function by determining the amino-acid sequence of polypeptides. The first demonstration of this, made by Vernon Ingram in 1956, showed that the substitution of one amino acid for another can be a matter of life or death.

Sickle Cell Anaemia

In normal humans the red blood cells contain a protein, haemoglobin A (HbA), which not only gives the red blood cells their colour but is essential for transporting oxygen around the body. In one particular inherited disease, sickle cell anaemia, the red blood cells, instead of being flat discs, are sickle shaped and are very inefficient oxygen carriers. The result is a progressive haemolytic anaemia usually resulting in early death. This very serious condition is caused by homozygosity for a mutant gene Hb^S. In 1949 Linus Pauling, twice a Nobel Prize winner, showed firstly that individuals with the disease (genotype $Hb^S\ Hb^S$) have no normal haemoglobin A in their red blood cells and instead have an abnormal haemoglobin (HbS) which has a lower negative charge than HbA; and secondly that the $Hb^A\ Hb^S$ heterozygous carriers of the disease have *both* HbA and HbS in their red blood cells, leading to mild sickling but not to anaemia; Pauling considered this to be a gene-controlled *molecular disease.*

The Structure of Haemoglobin

By this time Sanger had developed the techniques necessary for working out the amino-acid sequence of the insulin molecule and Ingram now applied these techniques to the haemoglobin molecular complex. The unravelling of this complex was no easy task as it contains nearly 600 amino acids compared to the mere fifty-one of insulin. Fortunately, however, it turned out that haemoglobin is made up of two identical alpha chains (each with 141 amino acids) and two identical beta chains (each with 146 amino acids) and by 1956 Ingram was able to show that the only difference between haemoglobin A and haemoglobin S is that at one particular site on one of the polypeptides Hb S carries a valine residue instead of the normal glutamic acid residue of HbA. Later work established that the substitution occurred at position 6 of the β chain and confirmed that there were no other differences. A mutation in a gene has resulted in the substitution of one amino acid for another in the polypeptide specified by that gene (table 11.2).

Note that the heterozygous $Hb^A\ Hb^S$ carriers have both HbA and HbS and have mild sickling of the red blood cells so that both alleles are expressed and produce their corresponding gene products. If only the clinically important symptom of haemolytic anaemia is considered, the gene for sickle cell anaemia appears to be completely recessive to the wild-type gene, but when sickling of

Table 11.2 Abnormal human haemoglobins

Residue number	Amino-acid residue in wild-type sequence		Variant haemoglobin amino-acid substitution	name
1	Val			
2	His	→	Tyr	Hb Tokuchi
.				
5	Pro			
6	Glu	→	Val	HbS (sickle cell haemoglobin)
		→	Lys	HbC
7	Glu	→	Gly	HbG
		→	Lys	HbC Georgetown
.				
.				
.				
26	Glu	→	Lys	HbE
27	Ala			
28	Leu	→	Pro	Hb Genova
.				
.				
.				
63	His	→	Tyr	HbM Saskatoon
		→	Arg	Hb Zurich
.				
67	Val	→	Glu	HbM Milwaukee
.				
.				
99	Asp	→	Asn	Hb Kempsey
		→	His	Hb Yakima
.				
.				
.				
136	Gly	→	Asp	Hb Hope

This table lists some of the variations of the haemoglobin β chain. The residues are numbered from the N terminal end. Most of the abnormal haemoglobins are named after the locality where they were discovered. Over 35 variants of the α chain and 65 of the β chain are known.

the red blood cells is considered Hb^S appears to be dominant to Hb^A. Strictly speaking the terms 'dominant' and 'recessive' should not be applied to the genes themselves but only to the characters they control, since a particular gene can control some characters that are dominant and others that are recessive; caution must always be exercised in interpreting a recessive character as due to the absence of a gene product rather than the presence of an altered product.

Many different variants of haemoglobin have been found and all differ from

the wild type HbA by a single amino-acid substitution in either the α or the β chain; some of these abnormal haemoglobins are shown in table 11.2.

In addition to HbS, due to the substitution of a glutamic acid by a valine residue at position 6 on the β chain, HbC is also due to the substitution of the same glutamic acid residue but by a lysine residue. Individuals with only HbC are homozygous *HbC HbC* but their only abnormality is a mild form of anaemia. Other abnormal haemoglobins cause varying symptoms, while some, for example Hb Hope due to a substitution at position 136 on the β chain, do not cause any observable clinical symptoms. Clearly, the ability of a polypeptide to assume a normal secondary and tertiary configuration and consequently to have normal biological activity, depends on having specific amino acids at some positions while at other positions the particular amino acid present is unimportant.

Tryptophan Synthetase

An even more precise definition of the relationships between a gene and the polypeptide it specifies comes from the work of Charles Yanofsky and his group on the enzyme tryptophan synthetase in *Escherichia coli*. Tryptophan synthetase converts indoleglycerol phosphate and serine into tryptophan and is composed of two different proteins, the A and B proteins, specified by two adjacent genes on the *E. coli* chromosome, *trpA* and *trpB*. The active enzyme complex consists of two A proteins and two B proteins, easily separable *in vitro*. The A protein consists of a single polypeptide chain 267 amino acids long and in a known sequence. The rationale behind these experiments was to isolate a number of mutants which produced defective A proteins, to construct a genetic map showing the locations of the mutations within the *trpA* gene, and to examine the amino-acid sequences in the A protein specified by each mutant. In this way they hoped to show what had been generally accepted for a number of years but was still unproven, that the gene and the polypeptide it specifies are co-linear.

They isolated a series of mutants with alterations at a number of different sites within the *trpA* gene; in particular, they looked for mutants producing an A protein which could still complex with the B protein but which had lost the ability to catalyse the reaction IGP + serine → tryptophan. A genetic map of these mutants was then constructed by phage-mediated transduction using methods similar to those described in chapter 7 and, finally, they determined the amino-acid changes in each of the mutationally altered proteins. Their results are summarised in table 11.3.

Particularly interesting are the substitutions at positions 210 and 233 in the A protein. At position 210, for example, the *trpA23* mutation causes the substitution of arginine for glycine, while *trpA46* causes the *same* glycine to be replaced by glutamic acid; the mutant sites in *trpA23* and *trpA46* are separable by recombination, although at a very low frequency. Since an amino acid is coded for by only three nucleotide pairs in the DNA, we are looking at a pair of mutant sites separated at the most by one nucleotide pair—in fact, from our knowledge of the genetic code (chapter 12) we know that the *trpA23* and

Table 11.3 Co-linearity of gene and polypeptide

The *E. coli* chromosome	Number and relative position of mutant site	Number of affected residue	A protein residue in wild type		mutant
	A3	48	Glu	→	Val
	A446	174	Tyr	→	Cys
	A487	176	Leu	→	Arg
	A223	182	Thr	→	Ile
	A23	210	Gly	→	Arg
	A46	210	Gly	→	Glu
	A187	212	Gly	→	Val
	A78	233	Gly	→	Cys
	A58	233	Gly	→	Asp
	A169	234	Ser	→	Leu

The results of Yanofsky's experiments on the tryptophan synthetase-A protein from *E. coli*. There is a direct correspondence between the order of the mutant sites within the *trpA* gene and the position of the amino acid replacement within the A protein.

trpA46 mutations have affected *adjacent* nucleotide pairs in the DNA so that this is genetic analysis taken to the theoretical limit of its resolving power.

These studies clearly show that (i) there is a complete correspondence between the sequence of mutational sites within the *trpA* gene and the positions of the amino acid substitutions within the A protein (this is what we understand by co-linearity), (ii) no mutation has affected more than one amino-acid residue, and (iii) different very closely linked mutations can cause different amino-acid substitutions at the same position in the A protein.

Intragenic Complementation

Earlier in this chapter and in chapters 7 and 9 we saw that complementation can be used to determine whether two independent mutations are located within the same or within different genes; if the mutations are in different genes they are expected to complement (*inter*genic complementation) while if they are two independent mutations within the same gene they will not complement. However, this simple relationship does not always hold as there are numerous examples in bacteria and fungi where two mutations affecting the *same* enzyme and mapping within the *same* gene can complement each other to form an active enzyme when they are combined together in the same cytoplasm—this is called *intra*genic complementation. At the experimental level the criterion for distinguishing between intergenic and intragenic complementation is that, whereas any two mutations that are in different genes will always complement

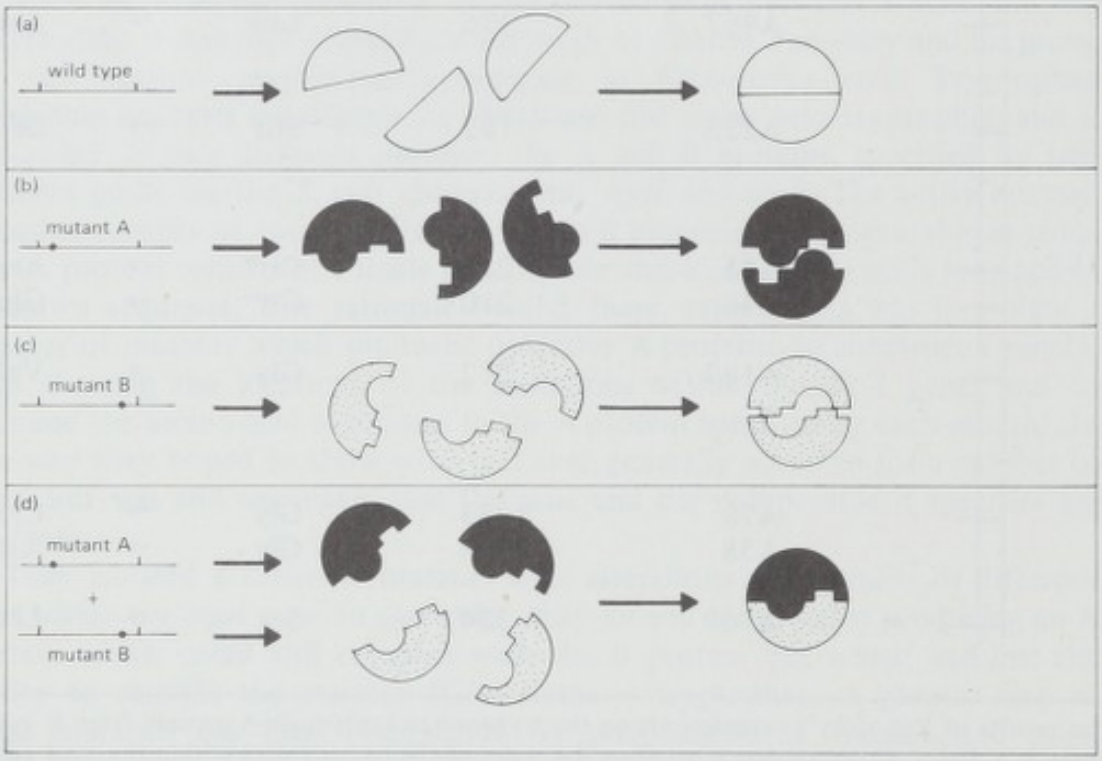


Figure 11.8 Intragenic complementation. The three shapes represent the three-dimensional configurations of the protein subunits produced by the wild type and by two mutant strains. An active enzyme can only be produced when two subunits are able to polymerise.

(a) The three-dimensional configuration of the protein subunits is such that they can polymerise to form an active enzyme complex (represented by the circle).

(b) The three-dimensional configuration has been changed so that the subunits can no longer polymerise to form an active enzyme.

(c) Another mutant may change the three-dimensional configuration in a different way; but the subunits again cannot polymerise to form an active enzyme.

(d) When both mutant genes are together in a heterocaryon, the two types of subunit may be able to polymerise and form an enzyme with reduced activity. Only 50 per cent of the pairs of subunits can complement—the other 50 per cent will be pairs of like subunits, which will be inactive.

each other, it is only certain exceptional pairs of mutants that show intragenic complementation—that is to say, most pairs of mutations occurring within the same genes will not complement.

In order to show intragenic complementation both mutants must usually (i) be point mutations and not deletions, and (ii) produce either a protein that is serologically related to the missing enzyme but without enzymic activity (this is called cross-reacting material or CRM) or a modified enzyme with very low activity. In other words the mutations involved must be missense rather than nonsense or frameshifts. Thus, although the temperature-sensitive mutants of T4 show intragenic complementation, the amber mutants do not. Furthermore, the enzyme produced by complementing mutants is qualitatively different from that produced by the wild type and has, at the most, 25 per cent of the activity of the wild-type enzyme.

One explanation of intragenic complementation lies in the fact that many proteins are aggregates of two or more identical polypeptide chains (table 11.1) so that if mutation causes an amino-acid substitution in this polypeptide subunit it may change the three-dimensional configuration of the subunit in such a way that it can no longer aggregate to form an active enzyme (figure 11.8). Two different allelic mutants will each produce an altered polypeptide, neither being able to aggregate with itself to form active enzyme; however, if these two mutations are combined together into a heterocaryon or F-prime merodiploid, the two differently altered subunits *may* be able to aggregate to form a molecule with some enzymatic activity. The easiest way of visualising this is to imagine that the two mutations have altered the subunits so that they have complementary shapes and can aggregate in dissimilar pairs (figure 11.8).

Intragenic complementation has been detected in many genes, for example the genes specifying the production of imidazolglycerol phosphate dehydrogenase in *Salmonella typhimurium*, of tryptophan synthetase in *Escherichia coli* and *Neurospora crassa* and of glutamic dehydrogenase in *N. crassa*.

The occurrence of intragenic complementation does not mean that we must abandon the concept of the gene as defined by complementation so long as we regard the inability of two mutants to complement as their failure to produce a *wild-type* protein.

Summary of Key Points

(1) Many proteins are macro-molecular complexes made up of aggregates of one or more protein subunits—these subunits are synthesised as polypeptide chains which become folded and finally aggregated to form the complex.

(2) Each type of polypeptide chain is specified by a different gene, and, apart from the genes involved in genetic regulation and the synthesis of RNA, each gene specifies a different polypeptide.

(3) Enzymes are proteins so that a mutation in a gene specifying an enzyme or one of its subunits can result in a biochemical defect, the organism being unable to carry out a particular step in a biosynthetic pathway.

(4) The sequence of different mutant sites within a gene corresponds to the sequence of amino-acid substitutions in the polypeptide specified by that gene—the gene and its product are co-linear.

(5) Although complementation between two mutants usually indicates that the two mutations are in different genes some pairs of allelic mutants also complement; this intragenic complementation is due to the interaction of the differently altered subunits so as to produce a partially active enzyme.

12 The Genetic Code

No question is ever settled
Until it is settled right.
Ella Wheeler Wilcox (1855-1919)

In previous chapters we have seen that a mutation in a structural gene may result in the substitution of one amino acid for another in a protein and it is clear that these genes act by specifying the primary structures of proteins. Since the genes are DNA and the only difference between one piece of DNA and another is in the particular sequences of nucleotides along the molecules, it follows that the amino-acid sequence of a protein must be determined by the sequence of nucleotides in the gene which specifies it—this is the *sequence hypothesis*, first stated by Crick in 1958; a corollary of this hypothesis is that the gene and the polypeptide it specifies should be co-linear (page 191). In this chapter we shall first consider the theoretical basis of a simple code and see how its general nature was established experimentally, and then see how it has been possible to determine the exact nucleotide sequences (or *codons*) which code for each of the twenty amino acids found in proteins.

Theoretical Aspects of the Code

Partly because the two strands of a DNA molecule are base complementary, and partly because messenger RNA is templated along only one of the two strands of a DNA molecule, it is both more convenient and more usual to talk about the sequence of bases along one strand of the DNA, or along the single strand of messenger RNA, rather than the sequence of base pairs along the DNA double helix; the four nucleotides A, T (or U), C and G can be regarded as the letters of the code.

The simplest type of code is one in which each of the twenty amino acids is encoded in a short sequence of nucleotides, a codon or codeword. If each codeword consists of only two letters the genetic language is restricted to sixteen (ie 4×4) codons (AT, AC, AG, AA, TT, TC, TG, . . . etc.), not enough to code for the twenty amino acids, while a three-letter or *triplet* code is more than adequate and provides sixty-four ($4 \times 4 \times 4$) different codewords and each amino acid is coded for by a sequence of three bases. Since there are twenty amino acids and sixty-four available codons, there may be some codons which do not code for any amino acid, while some amino acids may be specified by more than one codon; in the former case the code is said to be *redundant*, and in the latter it is *degenerate*.

Any code which is based on codewords of two or more letters can be either

overlapping or *non-overlapping*. For the moment let us assume a triplet code and consider the nucleotide sequence

ATCGCTATCTAG

If the code is non-overlapping the first triplet (ATC) will code for the first amino acid, the second triplet (GCT) for the next and so on; each consecutive triplet codes for one amino acid. On the other hand, with an overlapping code, ATC will again code for the first amino acid but the second amino acid will be coded for by TCG, the third by CGC and so on (figure 12.1). The final distinction is between a code with *commas* and one without. If the code has commas each codeword is separated from the next by a short untranslated nucleotide sequence so that there is a built-in mechanism to ensure that the code is correctly read. A code with commas can be represented

*ATC*GCT*ATC*TAG*

where ATC, GCT, etc, are the codons and the asterisks represent the nucleotide sequence of the commas; note that a code with commas cannot also be overlapping. With a *commaless* code there is a reading frame provided by the translational machinery of the cell (chapter 14) and the code is continuous and is read in consecutive groups of three from a fixed starting point. Thus the nucleotide sequence

ATCGCTATCTAG

must always be read as

ATC GCT ATC TAG

and not as

A TCG CTA TCT AG

or

AT CGC TAT CTA G

If the code is incorrectly read because reading commences at the wrong point, the result is a polypeptide with a hopelessly incorrect sequence of amino acids; we say that the reading frame has shifted and that the code is read out of phase.

As will be seen, the genetic code is a non-overlapping, commaless, degenerate triplet code read from a fixed starting point.

The Genetic Code is Non-overlapping

In early years it was recognised that each amino acid is coded for by at least three nucleotides, and the first formal scheme for a genetic code was proposed by George Gamow in 1954. He suggested that the code was completely overlapping so that there was a one-to-one relationship between the number of nucleotides and the number of amino acids; this explained very neatly the *apparent* correlation between the internucleotide distance (0.34 nm) and the distance between adjacent amino acids in a polypeptide chain (0.36 nm). However, there is now an imposing body of evidence against such an overlapping code. Firstly, it places severe restrictions on the sequence of amino acids in a

polypeptide. For example, with a completely overlapping code the codeword ATC *must* be followed by one of the four codewords beginning with TC, the next codeword must begin with C, and so on, and no such restrictions have ever been observed. Secondly, a mutation which changes one base for another would affect *three* adjacent amino acids in the corresponding protein (figure 12.1), instead of the one amino acid that is changed with a non-overlapping code. All the amino-acid substitution data (including substitutions in the haemoglobins and tryptophan synthetase-A protein described in chapter 11) indicate that only one amino acid is changed.

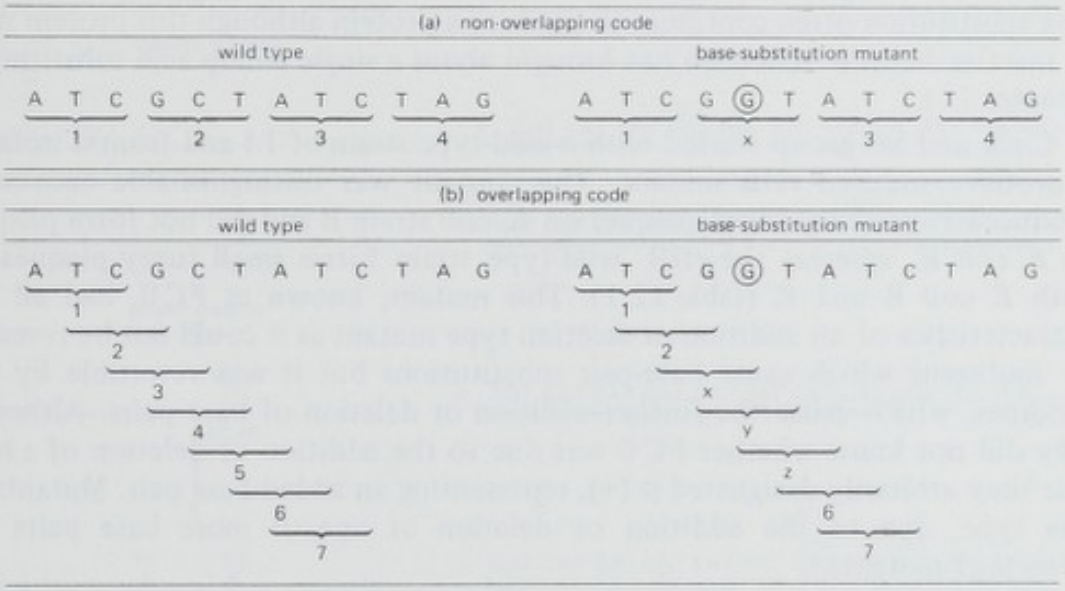


Figure 12.1 Non-overlapping and overlapping codes.
(a) With a non-overlapping code a single base substitution causes the replacement of *one* amino acid by another in the polypeptide specified by that gene.
(b) With an overlapping code three adjacent amino acids are altered in the polypeptide. The numbers refer to the amino acids in the wild-type sequence and the letters to the substituted amino acids.

The General Nature of the Genetic Code

The general nature of the genetic code (that is, it is a degenerate, commaless triplet code) was very elegantly established by a series of experiments carried out by Francis Crick, Leslie Barnett, Sydney Brenner and Richard Watts-Tobin in 1961, using the *r*IIB system of phage T4 first exploited by Seymour Benzer (chapter 9). The success of their experiments depended on two things. First, the mutants they used all mapped in the left 10 per cent of the *r*IIB gene and so, if the gene and its polypeptide are co-linear, affect the structure of one end of the presumptive *r*IIB protein. From Benzer's work they already knew that considerable changes could be made to the structure of the left-hand end of the *r*IIB gene without greatly affecting its activity and that the effects of certain

harmful mutations in this region could be suppressed by other mutations within the *r*IIB gene. It seemed that very considerable changes could be made to one end of the protein supposedly encoded by the *r*IIB gene without altering its function; they suspected that these different mutations might provide a way to investigate the general nature of the genetic code. The second reason was that they induced mutations using a group of related compounds, the acridines; whereas most chemical mutagens (chapter 10) alter the structure of DNA by causing base-pair substitutions, the effect of acridines is to cause the *addition* or *deletion* of a base pair in the DNA. In general, most addition and deletion mutants do not produce any detectable protein, whereas a gene mutated by a base substitution often continues to produce a protein although this protein may be inactive because mutation has brought about a single amino acid substitution (chapter 11).

Crick and his group started with a wild-type strain of T4 and from it isolated a proflavin-induced *r*IIB mutant. This mutant was distinguishable because it produced large sharp-edged plaques on *E. coli* strain B and did not form plaques on *E. coli* K, whereas the *r*IIB$^+$ wild-type strain forms small fuzzy plaques on both *E. coli* B and K (table 12.1). This mutant, known as FC 0, had all the characteristics of an addition or deletion type mutant as it could not be reverted by mutagens which cause base-pair substitutions but it was revertible by the acridines, which cause the further addition or deletion of base pairs. Although they did not know whether FC 0 was due to the addition or deletion of a base pair they arbitarily designated it (+), representing an added base pair. Mutants of this type, due to the addition or deletion of one or more base pairs are *frameshift* mutants.

When large numbers of FC 0 phages (figure 12.2(2)) were plated on *E. coli* K only the rare reversions towards the wild type could grow and form plaques; eighteen reversions were purified by plating on *E. coli* B and were found to

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Figure 12.2 The Crick experiment.

(a) The experimental results. FC 0 (designated (+)) was the original proflavin-induced *r*IIB mutant; FC 1 and FC 21 were isolated as suppressors of FC 0, and FC 40 as a suppressor of FC 1. The continuous line represents the left 10 per cent of the *r*IIB gene.

(1) The wild-type forms fuzzy plaques on *E. coli* strains B and K.

(2) FC 0 is an *r*IIB mutant, unable to form plaques on *E. coli* K.

(3) Spontaneous reversions on FC 0 were selected by plating on *E. coli* K. Most were pseudowilds, due to 'suppressor' mutations within *r*IIB. These suppressors mapped at the eight sites shown.

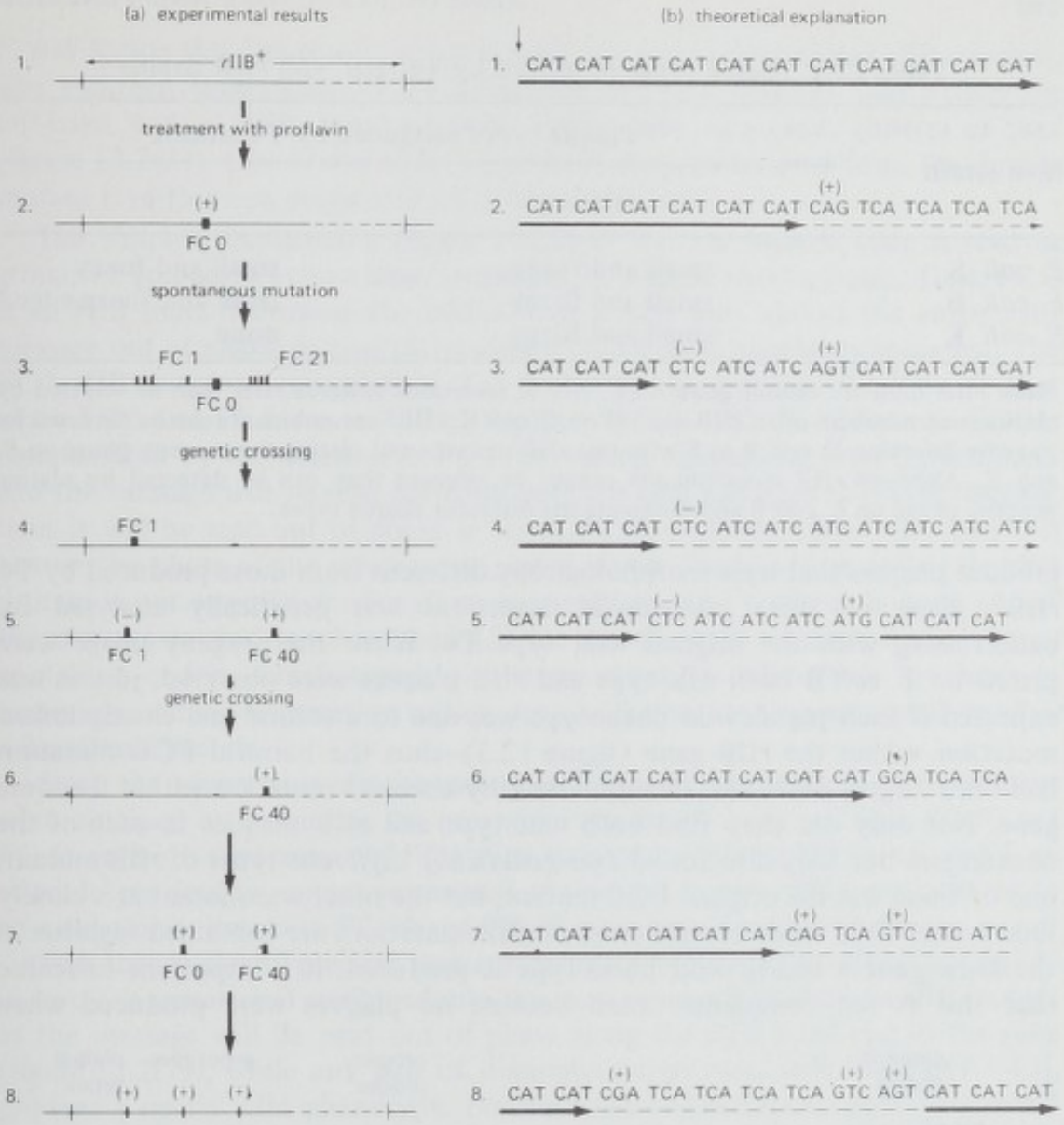
(4) The FC 1 mutation by itself was isolated by crossing the pseudowild with a wild-type strain. The FC 1 mutant has an *r*IIB phenotype.

(5) Suppressor reversions of FC 1 were isolated by plating FC 1 on *E. coli* K. These suppressors are designated (+).

(6) FC 40 by itself is an *r*IIB mutant.

(7) The double mutant FC (0 + 40) contains two base additions and is an *r*IIB mutant.

(8) A mutant with three base additions is a pseudowild.



(b) The theoretical explanation of these experiments. The solid and dashed lines represent the parts of the genetic message that are read in and out of phase respectively.

(1) The wild-type *r*IIB$^+$ sequence. The message is read in groups of three from a fixed starting point (↓).

(2) In the FC 0 mutant the message is read out of phase from the codon containing the added base to the end of the message.

(3) The suppressed reversion FC (0 + 1) has the FC 0 base-pair addition *and* a base-pair deletion at the FC 1 site. The only part of the message read out of phase is between the codons carrying the added and deleted bases.

(4) The FC 1 deletion mutation by itself is an *r*IIB mutant, since the right-hand end of the gene is read out of phase.

(5) FC (0 + 40) contains both a base deletion and a base addition and the only part of the message read out of phase is between the codons containing the added and deleted bases.

(6) FC 40 by itself, like FC 0, contains a base addition and is an *r*IIB mutant.

(7) The double mutant FC (0 + 40) contains two base additions and the message is read out of phase from the codon containing the first base addition.

(8) A mutant with three base additions will be read out of phase only between the first and last mutations, and the left-hand end of the protein will contain an extra amino acid.

Table 12.1 Plaque types produced by T4 *r*IIB$^+$ and *r*IIB strains

Host strain	Plaque types produced by T4 strains	
	*r*IIB$^+$	*r*IIB
E. coli S	small and fuzzy	small and fuzzy
E. coli B	small and fuzzy	large and sharp-edged
E. coli K	small and fuzzy	none

Since *r*IIB mutants cannot grow on *E. coli* K reversions towards *r*IIB$^+$ can be selected by plating vast numbers of an *r*IIB mutant on *E. coli* K. *r*IIB$^+$ recombinants can be detected by mixedly infecting *E. coli* B or S with two *r*IIB mutants and plating the progeny phage on *E. coli* K. Although *r*IIB recombinants cannot be selected they can be detected by plating progeny phage on *E. coli* B and observing the different plaque types.

produce plaques that were morphologically different from those produced by T4 *r*IIB$^+$. Each of these *pseudowild* reversions was genetically analysed by backcrossing with the original wild type T4. When the progeny phage were plated on *E. coli* B both wild-type and *r*IIB plaques were observed, just as was expected if each pseudowild phenotype was due to a second and closely linked mutation within the *r*IIB gene (figure 12.3)—thus the harmful FC 0 mutation had been largely corrected, or *suppressed*, by a second mutation within the same gene. Not only did they find both wild-type and *r*IIB plaques in each of the backcrosses but they also found *two genetically different* types of *r*IIB mutant; one of these was the original FC 0 mutant, but the other was mutant at a closely linked site. Thus when certain pairs of *r*IIB mutations are combined together in the same gene a nearly wild phenotype is produced. It is important to realise that this is not complementation because no plaques were produced when

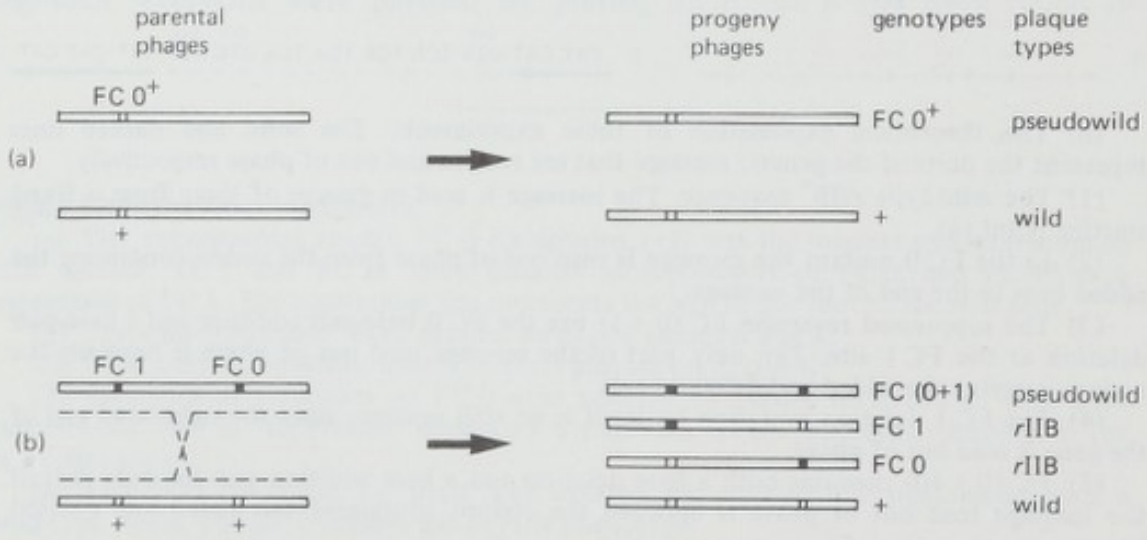


Figure 12.3 Reversion can be by a closely linked suppressor mutation. When the reversions of FC 0 are back-crossed to wild-type phage the progeny phenotypes will depend on whether the reversions are due to true back-mutation at the FC 0 site (a) or due to a second suppressor mutation within the *r*IIB gene (b). Only in the latter instance will *r*IIB progeny phages be recovered.

E. coli K was simultaneously infected with the pairs of individual *r*IIB mutants. The eighteen suppressors of FC 0 mapped at eight different sites within the left-hand end of *r*IIB, most of them falling into two close clusters of sites (figure 12.2(3)). One of the *r*IIB 'suppressor' mutants isolated from the double mutant reversion was designated FC 1 (figure 12.2(4)).

The simplest explanation (figure 12.2b) is that the genetic code is read in groups of (probably) three bases beginning at a fixed starting point. Thus, FC 0 is an *r*IIB mutant because the *addition* of a base puts almost the entire *r*IIB message out of phase, so that the reading of the code is hopelessly incorrect. The FC 1 *r*IIB mutation is explained if it is due to the *deletion* of a base, so that when FC 0 and FC 1 are combined together (FC(0 + 1)) the addition in FC 0 is compensated by the deletion in FC 1. If reading commences from the left-hand end the message will be read correctly until the first mutation (FC 1) is reached, then it will be read out of phase as far as FC 0, when the added base pair will restore the phase of the message. As noted above, the left-hand end of the *r*IIB protein is not essential for function, so provided that (1) the right-hand end of the message is read in phase, and (2) the out-of-phase segment does not now contain a codon, which signals the termination of polypeptide synthesis (a nonsense or chain-termination triplet, page 213), the double mutant will produce a functional protein.

Since the suppressors are themselves *r*IIB mutants suppressors of suppressors were obtained by extending the procedure (figure 12.2 (5) and (6)). FC 40 and FC 41 are both suppressors of FC 1, first isolated by plating FC 1 on *E. coli* K so as to detect doubly mutant suppressed strains, and because FC 1 was inferred to be a deletion (-) mutant, FC 40 and FC 41 must be addition (+) mutants.

This theory clearly predicts that any pair of 'like' suppressors (that is, two (+) or two (-) mutations) combined into the same gene will produce an *r*IIB mutant as the message will be read out of phase along the right-hand end of the gene (figure 12.2(7)), while any pair of dissimilar suppressors will, like FC(1 + 40), produce a nearly wild phenotype. Fourteen pairs of like suppressors were tested (for example, FC(0 + 40)) and all produced an *r*IIB phenotype. Thirty-four pairs of dissimilar suppressors were tested and all but nine produced nearly wild phenotypes. The remaining nine double mutants (for example, FC(0 + 88) and FC(9 + 41)) had *r*IIB phenotypes, probably because the added or deleted base generated one or more chain-termination triplets in the region between the two mutations. These experiments clearly show that the code is commaless, as otherwise any single base change would result in only one codon being misread (that is, there would be no reading frame to be put out of phase) and a (+) mutation would not be able to compensate for a (-) mutation.

Finally, the theory enabled Crick to test that the code is a triplet rather than a quadruplet code. With a triplet code, but not with a quadruplet code, some triple mutants of the types (+) (+) (+) and (-) (-) (-) will be pseudowild, having just one more or one fewer amino acid in the non-essential part of the protein; other triple mutants would have *r*IIB phenotypes because of the generation of unacceptable nonsense triplets. Five combinations were found to give pseudowild phenotypes when grown on *E. coli* K (figure 12.2(8)).

These experiments demonstrate that the code is probably a triplet, or less likely a hexuplet, code, that it is read from a fixed starting point and that it is commaless. Furthermore, since most of the out of phase triplets allow the gene to function, there can be comparatively few unacceptable nonsense triplets and most of the sixty-four codewords must code for one or other of the twenty amino acids and the code must be highly degenerate (as we shall see, sixty-one of the sixty-four possible triplets code for amino acids).

The Biochemistry of the Genetic Code

Although Crick's experiments established beyond any reasonable doubt that the genetic code was read in small groups of probably three bases from a fixed starting point, a major remaining problem was to crack the code, that is, to decide which particular nucleotide sequences, or codons, coded for each of the amino acids. The most direct way to crack the code would have been to determine both the nucleotide sequence of a gene and the amino-acid sequence of the polypeptide it specifies and to compare the two sequences, but until quite recently technical limitations made it impossible to determine such long nucleotide sequences so that more indirect methods had to be used.

These methods were made possible by the demonstration in 1960 that DNA itself is not the template for protein synthesis and that the genetic information on the DNA is transferred to a molecule of messenger RNA, which passes to the ribosomes and there acts as a template for protein synthesis (chapter 13), and, second, by the development of *in vitro* systems of protein synthesis.

In Vitro Protein Synthesis

The synthesis of proteins in cell-free systems is a very useful tool for studying the roles of the different components required for protein synthesis as *in vitro* synthesis allows the conditions to be very carefully controlled, making it possible to investigate the effects of different inhibitors and of leaving out one component from the reaction mixture. In a typical experiment cells from a rapidly growing culture of *E. coli* are collected by centrifugation and ground up in a mortar with finely powdered aluminium oxide to break open the cells and release the cell 'sap'. This 'sap' is treated with DNase to break down the bacterial DNA and then centrifuged to remove the heavier and more rapidly sedimenting cell wall and membrane components; the resulting supernatant contains ribosomes, transfer RNA, messenger RNA and the enzyme systems necessary for protein synthesis. This mixture is transferred to a test tube, fortified with the energy-rich compounds ATP and GTP and the twenty amino acids added, at least one of which is ^{14}C labelled. After incubation at 37° C trichloracetic acid is added to precipitate the protein and, after washing, the amount of radioactivity is measured. This measures the amount of protein synthesis by the amount of radioactive amino-acid precursors incorporated; it is necessary to measure

protein synthesis in this way as the actual amount of protein synthesised is too small to be measured directly.

In such a cell-free system protein synthesis continues for thirty to sixty minutes and is then arrested because the mRNA used as template has all been degraded, but synthesis can be reinstated by adding bulk RNA from *E. coli* or from some other source. It seems that the *E. coli* ribosomes can accept templates not only derived from *E. coli* itself but also those derived from other organisms including the viruses. Then in 1961 Marshall Nirenberg and Heinrich Matthaei made the remarkable discovery that not only natural mRNA but also enzymatically synthesised RNA would reinitiate protein synthesis. They reasoned that synthetic mRNA containing only one or two bases in a known ratio might stimulate the incorporation into protein of one or two specific amino acids, or might even make possible the synthesis of simple proteins containing only a few amino acids. They were able to synthesise a number of ribopolynucleotides by using the enzyme polynucleotide phosphorylase, which links nucleotide *di*phosphates in a random order—that is to say, it does not make RNA by following a DNA template.

One of the first ribopolynucleotides they synthesised contained only uracil and is known as polyU. When polyU was added to the cell-free system it stimulated the incorporation of just one amino acid, phenylalanine. To show this they carried out twenty separate experiments using polyU as an artificial messenger, and in each experiment a different one of the twenty amino acids was labelled, the remaining nineteen being unlabelled. Only when phenlalanine was labelled was there any significant incorporation of the ^{14}C label into protein. Thus one of the RNA codewords for phenylalanine is UUU. Similarly polycytidylic acid, PolyC, only stimulated the incorporation of proline so that CCC is a codon for proline.

Use of Random Polynucleotides

In a very short time two groups, led by Marshall Nirenberg and Severo Ochoa, had tested a wide range of synthetic ribopolynucleotides containing all possible combinations of A, U, C and G, and by comparing the relative amino-acid incorporation stimulated by different copolymers they were able to deduce the nucleotide *compositions* of many of the codons (but not the *sequences* of the nucleotides within the codons). Some of the experimental results and the calculated triplet frequencies in the ribopolynucleotides used are set out in table 12.2. Serine incorporation was stimulated by polyUC (a random *co*polymer of U and C) but not by polyUG, so its codon must be (UUC) or (UCC); it cannot be either UUU or CCC since these codons have already been assigned to phenylalanine and proline respectively (note that the brackets indicate that the nucleotide sequences within the codons are not known; thus (UUC) represents each of the sequences UUC, UCU and CUU). In polyUC (5 : 1) the frequency of each (UCC) codon is only 4 per cent, too low to account for the quite high level of incorporation observed (37 per cent) so that the most likely codon for serine is (UUC). By similar reasoning the probable codon for

Table 12.2 Deciphering the code using random ribopolynucleotides

polynucleotide composition		polyUC	polyUG	polyUGC
		5U:1C	19U:6G	34U:15G:5
Composition and frequency of each triplet	UUU	100	100	100
	UUC	20		147
	UCC	4		218
	CCC	0.8		322
	UUG		32	46
	UGG		11	21
	GGG		3	1
	CCG			101
	CGG			32
	UCG			68
Amino acid incorporation	Phe	100	100	100
	Ser	37	0	170
	Pro	12	0	188
	Leu	5	36	157
	Val	0	37	29.8
	Trp	0	14	1.6
	Gly	0	12	9.7

The triplet frequencies in each polynucleotide are relative to the frequency of UUU. In PolyUC the frequency of UUU triplets is 5/6 x 5/6 x 5/6 = 125/216, and the frequency of *each* of the three triplets containing 2U and 1C is 5/6 x 5/6 x 1/6 = 25/216, and relative to UUU the frequency of each (UUC) triplet is 25/125 x 100 = 20 per cent. The amounts of radioactive amino acid incorporated into protein are also expressed as a percentage of the total amount of phenylalanine incorporated.

Table 12.3 The genetic code

First letter	Second letter U	C	A	G	Third letter
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Term.	UGA Term.	A
	UUG Leu	UCG Ser	UAG Term.	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

The first letter of each of the sixty-four triplets is shown at the left, the second letter across the top, and the third letter down the right-hand side. The three triplets designated Term. are the chain-termination triplets. The table clearly illustrates how the degeneracy of the code is due largely to the unimportance of the base in the third position.

proline is (UCC), for valine (UUG) and for tryptophan and glycine (UGG). These deductions are confirmed by the third set of experiments using polyUGC; thus (UUC), allocated to serine, occurs with a frequency of 147 per cent and the amount of serine incorporated was 170 per cent; for valine (UUG) the corresponding figures are 21 per cent and 29.8 per cent, and so on. Note that

proline is coded by both CCC and (UCC) while leucine incorporation is stimulated by both polyUC and polyUG; since UUU codes for phenylalanine there must be at least two codons for leucine, one containing U and C and the other U and G. These experiments are a direct demonstration that the code is degenerate.

These deductions should be compared with the now fully established codon sequences set out in table 12.3.

Triplet Binding Experiments

In 1964 two new methods were introduced which rapidly led to the almost complete deciphering of the genetic code. In protein synthesis the amino acids do not just diffuse into the ribosomes, they are actively transported there by being attached to specific molecules of transfer RNA (tRNA). Not only does each sort of tRNA recognise and join to a particular amino acid but it also carries a specific nucleotide sequence, the anticodon, which recognises and binds

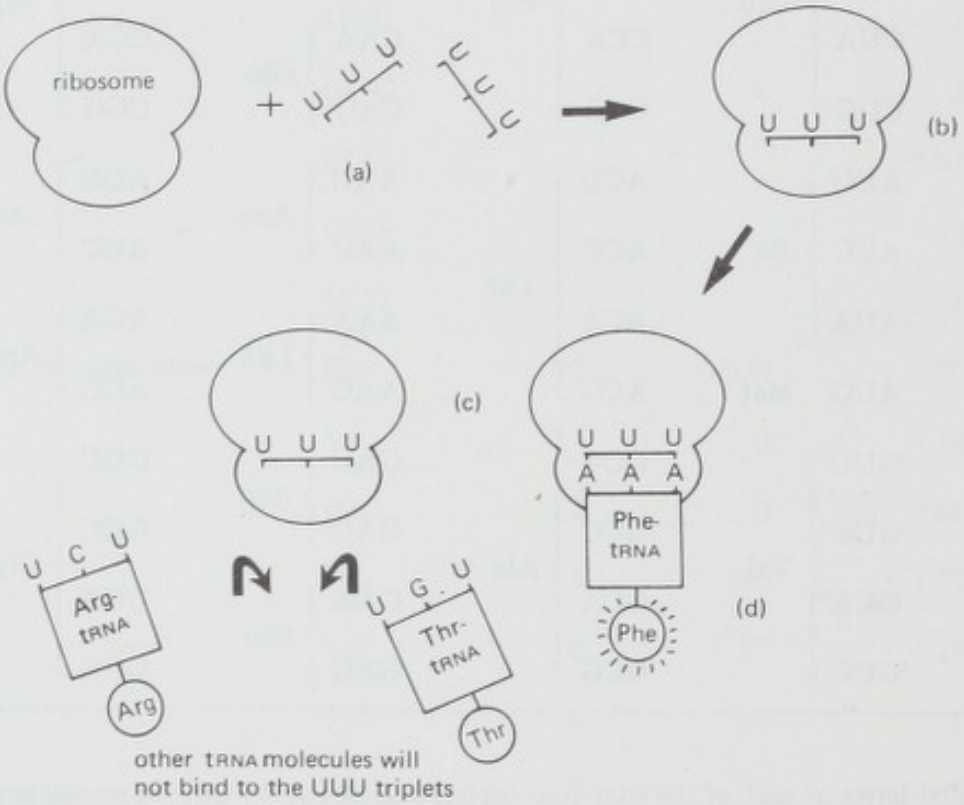


Figure 12.4 Triplet binding experiments.

(a) Specific nucleotide triplets (UUU) are added to an *in vitro* system containing all twenty amino acids, one of which is ^{14}C labelled.

(b) The triplets attach to the ribosomes.

(c) The triplets are recognised by the tRNA molecule with the correct anticodon and carrying one particular amino acid. Phe-tRNA is shown as binding to the UUU triplet.

(d) The mixture is passed through a nitrocellulose filter; this retains only the ribosomes and any bound amino acids. If the filter shows radioactivity then the nucleotide triplet used must code for the labelled amino acid. Which amino acid is bound is determined by repeating the experiment twenty times, using a differently labelled amino acid in each.

to the appropriate codon on the mRNA. Nirenberg and his co-workers found that when simple specific trinucleotides (that is, nucleotide triplets) were added to *E. coli* ribosomes they did not stimulate the incorporation of amino acids into protein but they did cause specific tRNA molecules with their attached amino acids to bind to the ribosomes. For example, pUpUpU caused phenylalanine to bind to the ribosomes. (It is conventional to write nucleotide sequences from the 5′ to the 3′ direction and pUpUpU indicates that the left-hand U has a phosphate attached in the 5′ position; UpUpUp indicates a phosphate attached to the right-hand U in either the 2′ or 3′ position.) They did this using the *in vitro* system already described by adding pUpUpU triplets instead of synthetic messenger RNA. The ribosomes were then separated by passing the mixture through a nitrocellulose filter which retained the ribosomes and any bound tRNA-amino acid complexes but allowed to pass through any unbound complexes; the binding of a particular amino acid was detected by the presence of the ^{14}C label in the ribosomes (figure 12.4).

There are three (UUG) codons, one of which we have already assigned to valine (page 205) and Nirenberg proceeded to identify each of these codons by binding experiments. He found that pGpUpU bound valine, pUpGpU bound cysteine and pUpUpG bound leucine. In these and other binding experiments nearly fifty of the sixty-four possible triplets gave unambiguous results and could be assigned to particular amino acids. The remaining triplets gave either very weak binding or else they bound more than one amino acid.

These experiments also showed that dinucleotides (for example pUpU) and trinucleotides with the phosphate in the 2′ or 3′ position (for example UpUpUp) were quite ineffective in binding, confirming that the coding ratio is probably 3 and suggesting that there is a polarity in the reading of the code.

Use of Regular Copolymers

The second method, developed by H. Gobind Khorana, was the most direct way of establishing the genetic code as he eventually succeeded in synthesising long mRNA molecules with regularly repeating sequences of bases. He used these molecules of strictly defined base sequence in an *in vitro* system and examined the amino-acid sequences in the resulting polypeptides. One copolymer he used was made by repeating the dinucleotide sequence UG, and this was found to stimulate the production of a polypeptide with alternating valine and cysteine. PolyUUG, made by repeating the trinucleotide sequence UUG, directed the synthesis of three separate homo-polypeptides, polycysteine, polyleucine and polyvaline. These results are just what are expected with a triplet code as the mRNA sequence UGUGUGUGUGUG . . . will be read in groups of three as UGU GUG UGU GUG . . . producing alternating UGU and GUG codons, and one of these must code for valine and the other for cysteine; a repeating trinucleotide such as polyUUG can be read either as UUG UUG UUG UUG . . . or as UGU UGU UGU UGU . . . or as GUU GUU GUU GUU . . . , depending on where the reading starts. Several polymers of repeating tetranucleotide sequences

Table 12.4 Regular copolymers and the genetic code

Messenger	Alternative reading frame positions						Polypeptides synthesised
$(UC)_n$	UCU	CUC	UCU	CUC	UCU	CUC...	Leu – Ser – Leu – Ser –
$(AC)_n$	ACA	CAC	ACA	CAC	ACA	CAC...	Thr – His – Thr – His –
$(AG)_n$	AGA	GAG	AGA	GAG	AGA	GAG...	Arg – Glu – Arg – Glu –
$(AAG)_n$	AAG	AAG	AAG	AAG	AAG	AAG...	Lys – Lys – Lys – Lys –
	AGA	AGA	AGA	AGA	AGA	AGA...	Arg – Arg – Arg – Arg –
	GAA	GAA	GAA	GAA	GAA	GAA...	Glu – Glu – Glu – Glu –
$(GUA)_n$	GUA	GUA	GUA	GUA	GUA	GUA...	Val – Val – Val – Val –
	AGU	AGU	AGU	AGU	AGU	AGU...	Ser – Ser – Ser – Ser –
	UAG	UAG	UAG	UAG	UAG	UAG...	none
$(UAUC)_n$	UAU	CUA	UCU	AUC	UAU	CUA...	Tyr – Leu – Ser – Ile –

In an *in vitro* system polynucleotides of defined sequence direct the synthesis of polypeptides of defined amino-acid sequence. Poly (GUA) produces only polyvaline and polyserine because UAG is a chain-termination triplet.

were also tested and some could stimulate the synthesis of a polypeptide with four alternating amino acids (table 12.4).

By themselves these results do not tell us precisely which triplet codes for which amino acid but when taken in conjunction with the results of the binding tests they are a remarkable confirmation of the code. The method also enabled several triplets to be assigned which had given inconclusive results in the binding tests. By the end of 1965, sixty-one of the sixty-four possible triplets had been assigned to particular amino acids (table 12.3). We now know that the three remaining triplets do not code for any amino acids but are chain-termination (or nonsense) triplets (page 213).

Confirmation of the Genetic Code

The experiments described so far studied protein synthesis and established the genetic code using *in vitro* systems, and although it seemed unlikely they did not rule out the possibility that a different code was used *in vivo*. However, the results of other experiments do demonstrate, almost with certainty, that the same code is used in both synthetic systems and in nature, and moreover that the same code is probably used in all organisms.

These approaches examined the consequences of different point mutations on polypeptide structure.

Amino-acid Substitution Data

The first approach to confirm the code was to examine the known amino-acid substitutions in *naturally* occurring proteins and to determine whether they were compatible with the code shown in table 12.3. If our deductions are correct and if the code is the same *in vivo* as *in vitro* then it should be possible to account for the overwhelming majority of the observed amino-acid substitutions by a *single* base-pair change in the DNA, that is by a single base change in the mRNA codon. For example, in haemoglobin M Saskatoon (table 11.2) the substitution of tyrosine for histidine occurs at position 63 in the beta chain. The inferred codons for histidine are CAU and CAC and those for tyrosine are UAU and UAC; a C → U change in the 5′C nucleotide of either histidine codon will generate one of the codons for tyrosine. Likewise, the change A → G will convert CAU or CAC into CGU or CGC respectively, two of the codons for arginine. This change, histidine → arginine, has been observed in Haemoglobin Zurich also at position 63 on the beta chain. On the other hand neither CAU nor CAC

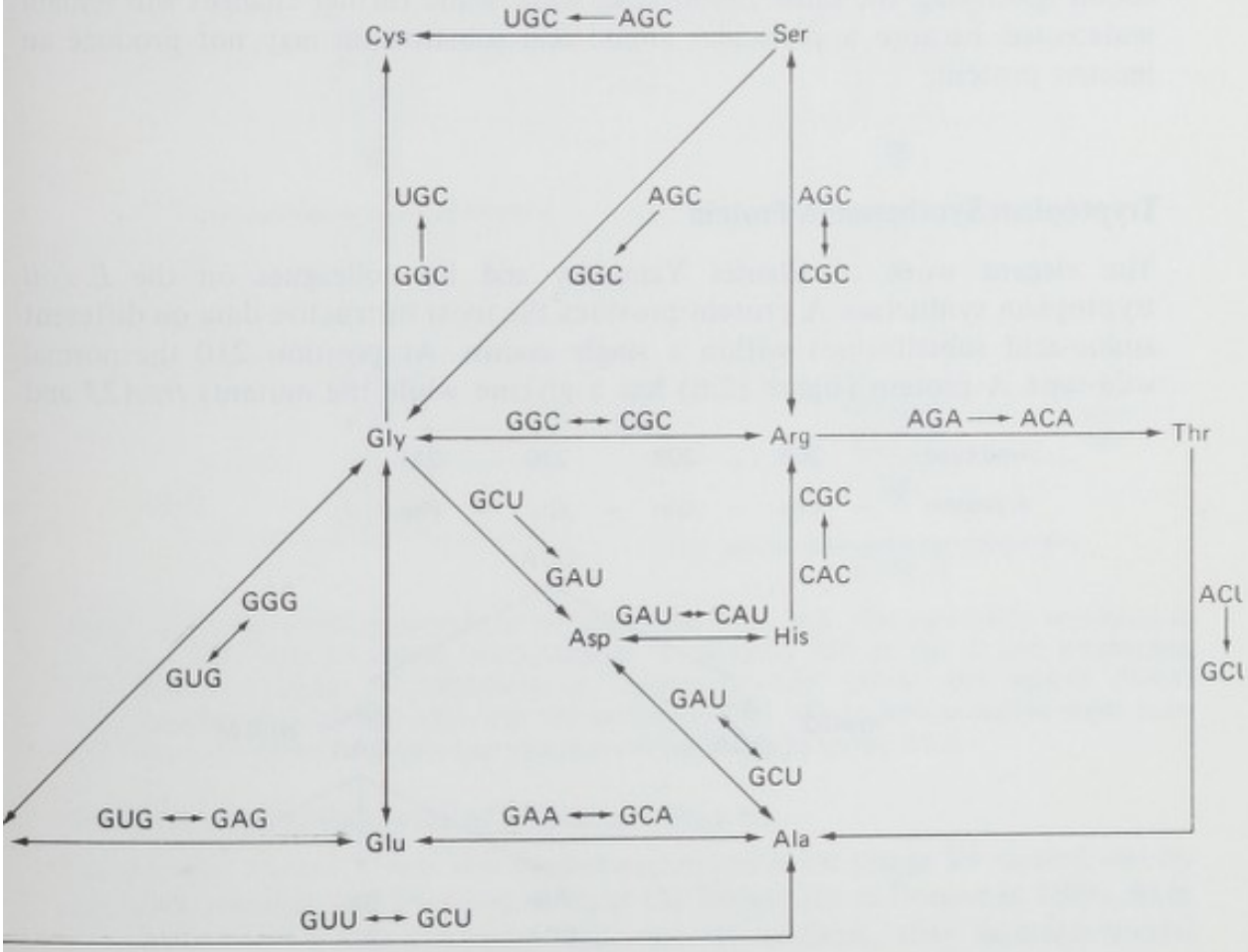


Figure 12.5 Amino-acid substitutions. Some of the amino-acid substitutions observed in the human haemoglobins, the *E. coli* tryptophan synthetase A protein, and the TMV coat protein. Many of the substitutions have been observed in more than one protein. All these substitutions can be accounted for by a single base substitution; *one* of the possible base substitutions to account for each change is shown.

(histidine) can change by a single base substitution into either AGU or AGC, the codons for serine, so that if our deductions are correct we do not expect to observe the substitution of serine for histidine or vice versa; these substitutions have never been observed. Figure 12.5 shows some of the observed amino-acid substitutions and *one* of the possible base substitutions that can account for each amino-acid substitution.

Since there are twenty different amino acids there are 20 × 19 or 380 possible amino-acid substitutions, but because of the restrictions just described there are only 136 possible amino-acid substitutions that can be accounted for by a single base change; a majority of these has already been described. Only one of the many observed amino-acid substitutions is contrary to our predictions. This is the change glutamic acid → methionine found by Yanofsky in the tryptophan synthetase A protein; this substitution was only recorded once and is almost certainly the rare instance of a double change (GAA or GAG → AUG).

Nearly one-quarter of all the possible base-substitution mutations will remain undetectable because their only effect will be to change a codon into another codon specifying the same amino acid, while some further changes will remain undetected because a particular amino-acid substitution may not produce an inactive protein.

Tryptophan Synthetase A Protein

The elegant work of Charles Yanofsky and his colleagues on the *E. coli* tryptophan synthetase A protein provides the most instructive data on different amino-acid substitutions within a single codon. At position 210 the normal wild-type A protein (figure 12.6) has a glycine while the mutants *trpA23* and

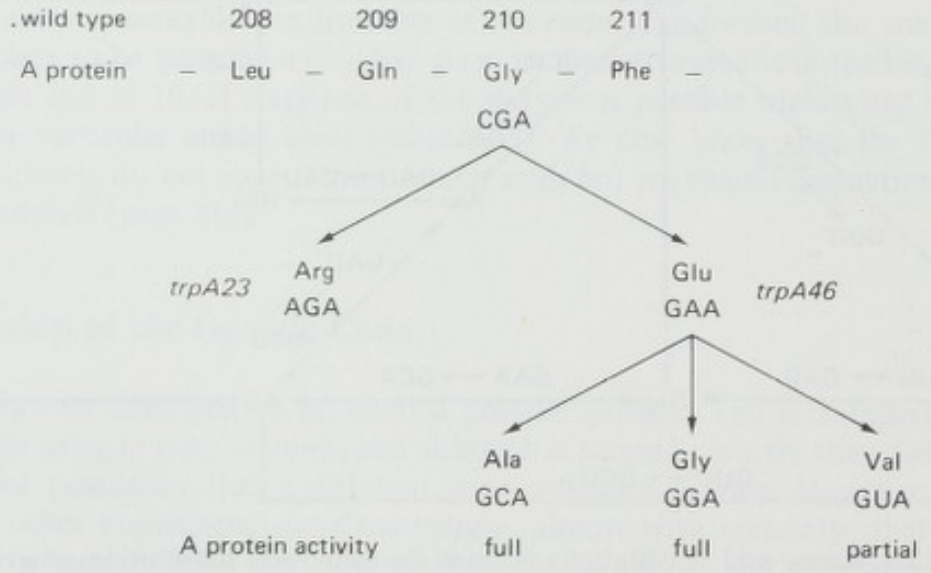


Figure 12.6 Several amino-acid substitutions can occur at the same position in a protein. Three different amino-acid substitutions have been observed at position 210 in the *E. coli* tryptophan synthetase A protein. The inferred codon changes are also shown.

trpA46 have arginine and glutamic acid respectively and have functionally inactive proteins. Yanofsky examined forty-seven reversions of *trpA46* towards wild type. In twenty-five instances the wild-type glycine had been restored and the A protein was fully active. In fourteen reversions the glutamic acid had been replaced by alanine and this protein was also fully functional. In eight instances the glutamic acid was replaced by valine producing a partially active A protein. These changes and the inferred codons are shown in figure 12.6. Note that the three types of reversion of *trpA46* are due to different substitutions of *one particular nucleotide* so that all the possible base substitutions at this nucleotide position have been identified.

By transduction experiments Yanofsky was also able to demonstrate a low frequency of recombination between *trpA23* and *trpA46*, and the wild-type transductants had a glycine at position 210. This shows genetic recombination at its highest possible resolution as the wild-type transductants could only have arisen by recombination between adjacent nucleotide pairs (figure 12.7). This was the first demonstration that recombination could occur within a triplet.

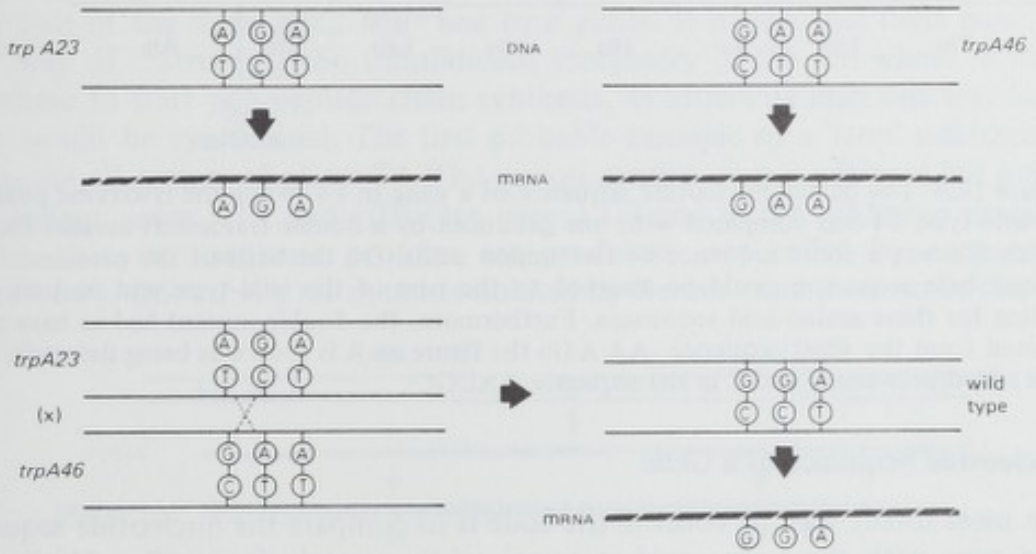


Figure 12.7 Nucleotide sequences in the *trpA* gene of *E. coli*. The nucleotide sequences in the DNA and mRNA codons corresponding to position 210 in the *E. coli* tryptophan synthetase A protein. In transductions between *trpA23* (AGA) and *trpA46* (GAA) recombination restores the wild-type codon (GGA); for this to have occurred there must have been a cross-over between two adjacent nucleotide pairs in the DNA.

Partial Nucleotide Sequence of a Gene in Phage T4

The second approach was the elegant experiments on phage T4 carried out by George Streisinger and his co-workers at the University of Oregon in 1966. As in the case of Crick's experiments with the *r*II mutants, they isolated several proflavin induced frameshift mutants in the gene specifying the phage lysozyme, and they were able to construct a double mutant with only a slight defect in the lysozyme; this mutant was presumed to carry one addition (+) mutation and one deletion (–) mutation. When the proteins were examined the wild-type and mutant lysozymes were found to be identical except for one short sequence of

five amino acids. Using the genetic dictionary (table 12.3) they were able to assign a unique sequence of fifteen bases for the message of the wild-type gene that codes for this short amino-acid sequence and to identify the bases involved in these (+) and (–) mutations (figure 12.8). Not only does this experiment confirm the genetic dictionary but it shows that the genetic message is read in the 5′ to 3′ direction as the protein is made from the N terminal to the C terminal end.

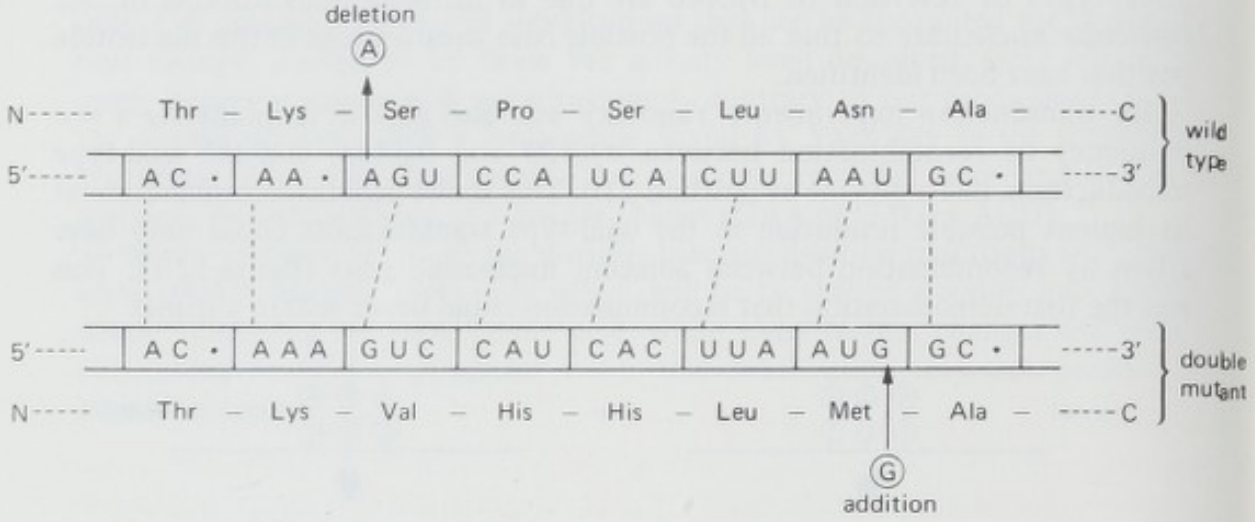


Figure 12.8 The partial nucleotide sequence of a gene in T4. When the lysozyme produced by wild-type T4 was compared with one produced by a double frameshift mutant the only difference was a short sequence of five amino acids. On the basis of the presumed code, unique base sequences could be assigned to the part of the wild-type and mutant genes coding for these amino-acid sequences. Furthermore, the double mutant had to have a base deleted from the short sequence ·AA·A (in the figure an A is shown as being deleted), and a G is added between U and C in the sequence AAUGC·.

Nucleotide Sequence of a Gene

The most direct way to confirm the code is to compare the nucleotide sequence of a gene with the amino-acid sequence of the protein it specifies. Until quite recently it was not possible to sequence long pieces of nucleic acid, but now single-stranded RNA, and to a lesser extent single-stranded DNA, can be enzymatically degraded into *specific* fragments small enough to be sequenced; by using different enzymes the RNA can be broken down into a series of overlapping fragments which, it is hoped, can then be arranged to yield a unique nucleotide sequence. Obviously to do this large amounts of specific RNA are required, and the most favoured material has been the RNA of the very small coliphages MS2, R17 and Qβ which not only functions as the gentic material but also as messenger RNA. These phage chromosomes, easily replicated in an *E. coli* host, are very small, only about 3300 nucleotides long, and contain just three genes coding for the phage-coat protein, the 'A' protein required for the phage to absorb on to the host bacterium and a synthetase required for the self-replication of the phage RNA. Large tracts of MS2, R17 and Qβ RNA have been sequenced in this way but one of the most striking achievements of 1972

was the determination of the complete nucleotide sequence of the coat-protein gene of MS2 by Walter Fiers and his colleagues W. Min Jou, G. Haegeman and M. Ysebaert working in Ghent. These experiments are further discussed in chapter 14 and some of this nucleotide sequence and the corresponding amino-acid sequence in the coat protein is shown in figure 14.11.

These experiments confirm in the most direct and the most exact way possible the codon assignments deduced from *in vitro* systems. We can note that in MS2 all sixty-one of the amino-acid codons have been found, forty-nine of them occurring in the coat-protein gene.

Chain-Termination Triplets

Polypeptide chains are assembled using a molecule of mRNA as an assembly template, but one molecule of mRNA may be the template for several different polypeptides; for example, in *E. coli* the mRNA molecule which encodes the tryptophan synthetase A protein (that is, the transcript of the *trpA* gene) also encodes the B protein and three other enzymes involved in protein synthesis (the transcripts of the *trpB*, *trpC*, *trpD* and *trpE* genes. It is clear that there must be some way of instructing the translational machinery of the cell where to stop and where to start polypeptide chain synthesis, as otherwise only one very long chain would be synthesised. The first probable example of a 'stop' mechanism was in the *r*II region of phage T4. This region consists of two adjacent but quite independent genes *r*IIA and *r*IIB (see page 137) and in the wild-type strain it appears that two separate proteins are produced, one coded by each gene (figure 12.9). One of the *r*II mutants isolated by Benzer turned out to be due to

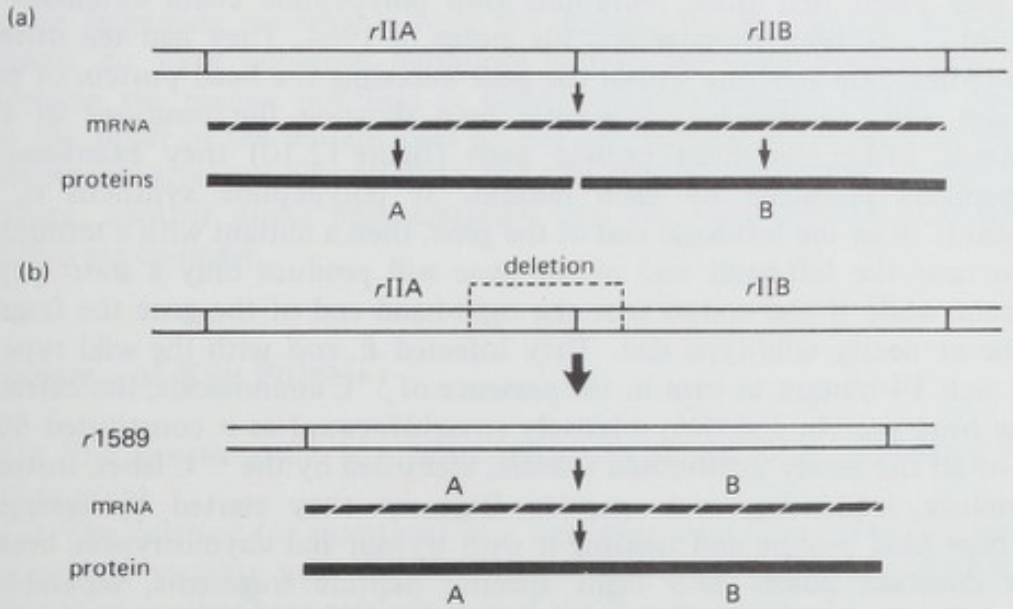


Figure 12.9 The *r*1589 deletion mutant of phage T4. The wild-type T4 *r*II region probably produces one molecule of mRNA which specifies both the *r*IIA and *r*IIB proteins (a). The *r*1589 deletion (b) is missing an essential part of the A gene, a non-essential part of the B gene and the presumed chain termination triplet at the end of the A gene. The result is a single protein molecule with some B activity but no A activity.

the deletion of a large part of the right-hand end of the *r*IIA gene, the 'join' between the genes and a smaller part of the left-hand end of the *r*IIB gene; complementation tests showed that this deletion mutant (*r*1589) produced no active *r*IIA gene product but that it did produce an active *r*IIB product. Benzer suggested that the deletion had removed a specific stop codon which marked where synthesis of the *r*IIA gene product was to terminate and the synthesis of the *r*IIB product to begin so that the messenger RNA was now read as a single continuum and in turn produced a long polypeptide chain corresponding to part of the *r*IIA protein plus enough of the *r*IIB protein to have some *r*IIB activity (Crick's experiments, described earlier in this chapter, later confirmed that the amino-acid sequence of the left-hand end of the *r*IIB protein was probably unimportant). It turned out that the three unassigned triplets code for chain termination, and are the so-called *nonsense* triplets, UAG (often called 'amber'), UAA ('ochre') and UGA.

Although chain-termination triplets must occur at the end of each gene, many mutant strains are the consequence of chain-termination triplets occurring in the middle of a gene, for in just the same way that mutation can change a codon for one amino acid into a codon for a different amino acid, so it can change a codon for an amino acid (a 'sense' codon) into a chain-termination codon (for example UGG tryptophan → UAG chain termination); this will result in the production of a polypeptide fragment as chain growth will cease when the translational mechanism reaches the termination codon.

The Head Protein Mutants of Phage T4

The first proof that these mutations stop polypeptide chain extension was obtained by Sydney Brenner and his group in 1964. They had ten different chain-termination mutants within the gene encoding the head protein of phage T4, and after constructing a genetic map showing the positions of these mutations within the head protein gene (figure 12.10) they examined the polypeptides produced by each mutant. If polypeptide synthesis is, say, rightwards from the left-hand end of the gene, then a mutant with a termination codon near the left-hand end of the gene will produce only a short peptide fragment while if the codon is at the right-hand end of the gene the fragment will be of nearly wild-type size. They infected *E. coli* with the wild type and with each T4 mutant in turn in the presence of ^{14}C amino acids; the extraction of the head protein was then relatively straightforward as it constituted 80 per cent of all the newly synthesised protein, identified by the ^{14}C label. Instead of laboriously sequencing each peptide fragment they started by taking the wild-type head protein and treating it with trypsin and chymotrypsin, breaking it at constant points into eight specific peptide fragments, separable by electrophoresis (see page 320). It was then simple to detect which fragments were missing from each T4 mutant, and so if and where peptide synthesis had terminated. They found that, first, the nearer the mutant site to the left end of the gene the fewer the number of peptide fragments produced (figure 12.10); and secondly, the mutant sites can be ordered according to the number of

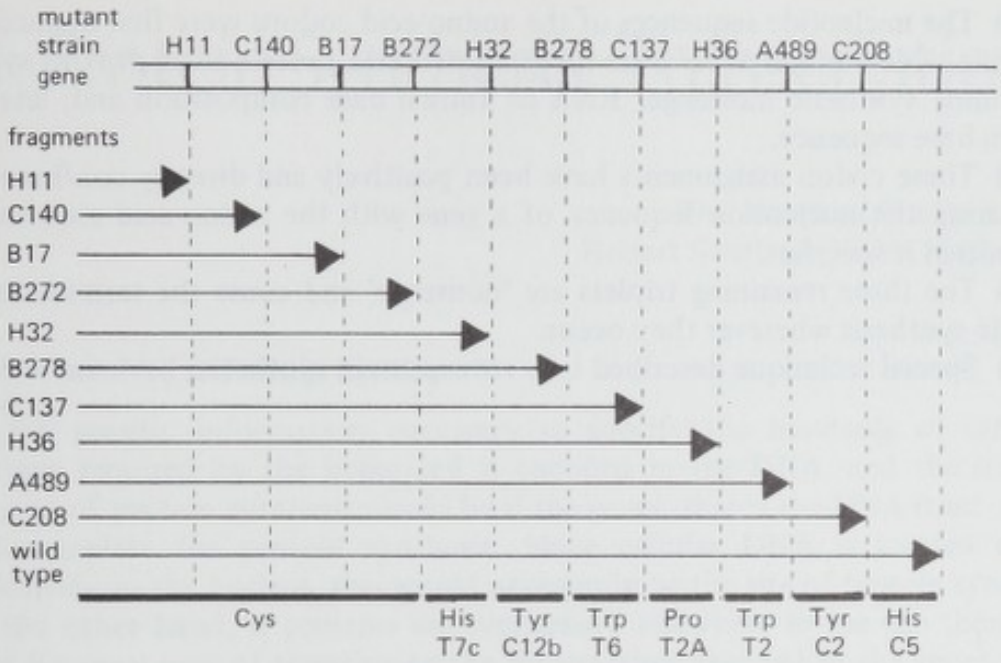


Figure 12.10 Chain-termination triplets in the T4 head protein gene. The top line shows the map positions of ten different head-protein mutants. Enzymatic digestion of the wild-type head protein generated the eight peptide fragments shown at the bottom of the figure. These fragments ('Cys' to 'HisC5') correspond to the entire head-protein molecule. The lengths of the peptide chains produced by each mutant (arrows) was determined by seeing which peptide fragments were missing from each mutant head-protein polypeptide. The mutants B17, C140 and H11 did not produce a complete peptide fragment but progressively shorter pieces of the 'Cys' peptide. The fragments can be arranged in a hierarchical order from left to right.

peptide fragments recoverable from the infected bacteria. It follows that these mutants do have nonsense mutations within the head-protein gene, which determine the precise positions at which chain termination occurs. Note also that the order of the mutant sites on the genetic map is the same as when the mutants are arranged according to the size of the polypeptide fragments they produce, another direct demonstration that the gene and the polypeptide it specifies are co-linear.

Summary of Key Points

(1) The genetic code is a non-overlapping triplet code read from a fixed starting point.

(2) The deletion (or insertion) of a base pair within the nucleotide sequence of a genetic message puts the code out of phase; the subsequent insertion (or deletion) of another base pair can restore the reading phase.

(3) The alteration of one base pair for another may cause the substitution of one amino acid for another in the polypeptide encoded by that gene.

(4) Sixty-one of the sixty-four possible codons code for the twenty amino acids commonly found in proteins; thus the code is highly degenerate.

(5) The nucleotide sequences of the amino-acid codons were first deduced by studying which amino acids were incorporated into protein in an *in vitro* system containing synthetic messenger RNA of known base composition and, later, of known base sequence.

(6) These codon assignments have been positively and directly confirmed by comparing the nucleotide sequence of a gene with the amino-acid sequence in the protein it specifies.

(7) The three remaining triplets are 'nonsense' and cause the termination of peptide synthesis wherever they occur.

(8) Special technique described is *in vitro* protein synthesis.

13 Protein Synthesis: Transcription

The March of Intellect.
Robert Southey (1774–1843)

RNA is Involved in Protein Synthesis

All the genetic information necessary to specify the hundreds of different proteins required by the living cell is encoded in the DNA, and the simplest concept of protein synthesis would be if the genes, that is the DNA itself, served as a template for protein synthesis. Since cellular DNA is located almost exclusively in the nucleus, this would necessarily be the site of protein synthesis. On the other hand, if proteins are synthesised elsewhere in the cell then there must be some way of transferring the information encoded in the genes to the actual site of protein synthesis. One of the first and most interesting approaches to this problem was the work of Joachim Hämmerling on the unicellular alga *Acetabularia mediterranea. Acetabularia* may grow up to 5 cm long and through most of its life-cycle it is differentiated into a chloroplast-containing stalk and a root-like base containing the single nucleus; later, the tip of the stalk is further differentiated into an umbrella-like cap concerned with reproduction. Stalks that have had the nucleus removed not only survive for a limited period but may also form large caps, and as early as 1934 Hämmerling concluded that this was possibly because of 'nucleus-dependent morphogenetic substances' stored in the stalk. Later experiments by Hämmerling and by Jean Brachet showed that the enucleate cytoplasm can still synthesise protein. Quite clearly the nucleus is not necessary for continued protein synthesis and the information on the genes must be transferred to some cytoplasmic substance. Further experiments by Brachet (1955) using onion root tips and *amoebae* showed that protein synthesis ceases if the cellular RNA is broken down by adding RNase and that the further addition of RNA extracted from yeast reinitiated a limited amount of protein synthesis. In the same year Lester Goldstein and Walter Plaut labelled the nuclear RNA of *Amoeba proteus* and transplanted the labelled nuclei into non-radioactive *amoebae.* They followed the distribution of the label by autoradiography (see pages 31 and 58) and observed that a large fraction of the labelled nuclear RNA subsequently moved into the cytoplasm, a direct demonstration that RNA is synthesised in the nucleus and that after synthesis many of these molecules move into the cytoplasm where protein synthesis occurs. These results all strongly supported the view that RNA is involved in protein synthesis.

Proteins are Synthesised on the Ribosomes

Most of the RNA in a cell is located in numerous small bodies, the ribosomes,

each about 20 nm in diameter. In *E. coli* there may be up to 15 000 ribosomes packed throughout the cytoplasm of each cell, making up one-quarter of the cell mass. In higher organisms some of the ribosomes exist free in the cytoplasm but mostly they line the outer surface of the endoplasmic reticulum (figure 1.4). In 1955 J. W. Littlefield fed ^{14}C-labelled leucine to rats and after a short time killed the rats, broke open the liver cells and separated the cellular components by fractional centrifugation. He found that the larger part of the ^{14}C label had been incorporated into protein and was associated with the ribosomes, the first indication that the ribosomes were the actual sites of protein synthesis. With this discovery it seemed possible that the ribosomal RNA itself (rRNA) carried the transcript of the genes, but several reasons made this unlikely. First, the base composition of rRNA bears no relationship to the base composition of DNA (table 13.1), and any polynucleotide, whether single- or double- stranded, which is formed by templating against DNA must have a base composition which reflects the base composition of its DNA template. Second, cells are able to switch enzyme synthesis on and off very rapidly, so that the templates on which the proteins are assembled must be short lived or unstable; rRNA is not short lived and can persist for at least three generations.

Table 13.1 The base composition (per cent) of *E. coli* and T2 DNA and RNA

	Escherichia coli			T2	T2 post-infection
	DNA	rRNA	total tRNA	DNA	DNA
A	25	25	21	32	31
U or T	25	21	21	32	29
G	25	32	28	18	18
C	25	22	31	18	22*

*5-hydroxymethylcytosine instead of cytosine.

The base composition of the RNA found in *E. coli* after T2 infection reflects the base composition of T2 DNA, and not that of *E. coli* DNA. The base compositions of *E. coli* DNA, rRNA and tRNA are apparently unrelated.

Messenger RNA

When phage T2 infects *E. coli* there is a very rapid halt to the synthesis of bacterial RNA and protein, followed rapidly by the synthesis of phage protein; since the phage injects only DNA into the bacterial cell this DNA must be able to create the specific templates required for the synthesis of phage protein. In 1957 Elliot Volkin and Lazarus Astrachan found that immediately after phage infection of the bacteria there is the rapid synthesis and equally rapid breakdown of a small amount of RNA, and that this RNA had base ratios that

reflected those of T2 DNA rather than of bacterial DNA (table 13.1). In 1961 François Jacob and Jacques Monod coined the term 'messenger' for the substance that transferred the genetic information from the DNA to the ribosomes and predicted that when isolated it would (1) be a polynucleotide; (2) have an average molecular weight of not less than 5×10^5 –enough to carry the code for an average sized gene, assuming a coding ratio of 3; (3) have a base composition reflecting the base composition of DNA; (4) be found, at least transitorily, associated with the ribosomes; (5) have a high rate of turnover. The rapidly turning over RNA observed by Volkin and Astrachan seemed to meet all these qualifications and Jacob and Monod designated it *messenger* RNA or mRNA.

Even before Jacob and Monod had published their ideas the concept of mRNA was confirmed by an elegant series of experiments carried out by Brenner, Jacob and Meselson (figure 13.1). In one of their experiments they

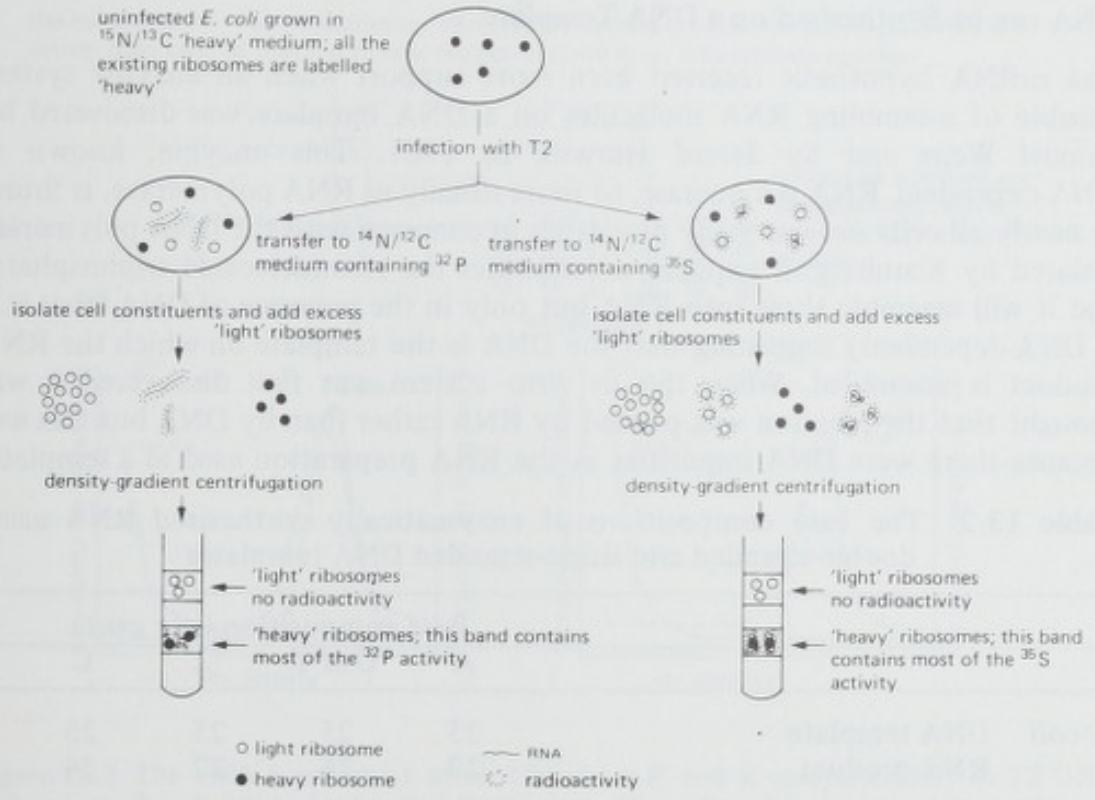


Figure 13.1 The ribosomes are for hire. The existing ribosomes of *E. coli* were labelled 'heavy' and 'cold' by growing the cells in heavy medium. After infection with T2 the cells were transferred to light medium containing either ^{32}P or ^{35}S. In the first experiment any new RNA appearing in the infected cells was labelled with ^{32}P. The cells were broken open, the ribosomes separated and density gradient centrifuged in the presence of excess 'light' and 'cold' ribosomes; it was found that nearly all the ^{32}P label was associated with the 'heavy' ribosomes. Thus the new RNA was associated with the pre-existing ribosomes. In the second experiment the light medium contained ^{35}S to label any new ribosomes and viral protein; again, most of the radioactivity was associated with the old ribosomes. Clearly, the viral protein must be synthesised by the pre-existing ribosomes which must direct protein synthesis according to the specificity of the mRNA with which they are associated. No new 'light' and 'hot' ribosomes were found.

grew uninfected cells of *E. coli* in $^{15}N/^{13}C$ medium so that all the nucleic acid and protein was uniformly labelled 'heavy'. After infection with phage T2 the cells were transferred to $^{14}N/^{12}C$ medium and fed with either ^{32}P (to label nucleic acid) or ^{35}S (to label protein) so that any newly synthesised cellular constituents were 'light' and 'hot'. Finally, the ribosomes were isolated and the old 'heavy' and any new 'light' ribosomes separated by spinning in a caesium chloride density gradient. The result of this experiment was that after phage infection the new phage specific RNA (labelled with ^{32}P) and the phage proteins (labelled with ^{35}S) were found to be associated with the pre-existing 'heavy' ribosomes previously used for the synthesis of bacterial proteins; no new 'light' and 'hot' ribosomes were found. The inescapable conclusion is that the ribosomes are 'for hire' and will direct protein synthesis according to the specificity of the mRNA that occupies them.

RNA can be Synthesised on a DNA Template

The mRNA hypothesis received even more support when an enzyme system capable of assembling RNA molecules on a DNA template was discovered by Samuel Weiss and by Jerard Hurwitz in 1961. This enzyme, known as DNA-dependent RNA polymerase, or more usually as RNA polymerase, is found in nearly all cells and has many properties in common with the DNA polymerase isolated by Kornberg; it requires as substrates the ribonucleoside triphosphates and it will assemble these into RNA but only in the presence of DNA (that is it is DNA-dependent) suggesting that the DNA is the template on which the RNA product is assembled. When this *in vitro* system was first discovered it was thought that the reaction was primed by RNA rather than by DNA but this was because there were DNA impurities in the RNA preparation used as a template.

Table 13.2 The base compositions of enzymatically synthesised RNA using double-stranded and single-stranded DNA templates

		Base composition (per cent)			
		A	U or T	G	C
E. coli	DNA template	25	25	25	25
	RNA product	23	26	27	24
T2	DNA template	32	32	18	18
	RNA product	33	31	18	18
X174	DNA template (single-stranded)	25	33	23	19
	RNA product	32	25	20	23

The RNA synthesised on double-stranded DNA primers has the same base composition as the DNA primer as *in vitro* both strands act as templates for RNA synthesis. A single-stranded DNA template yields RNA with an exactly complementary base composition as a hybrid DNA–RNA duplex is produced.

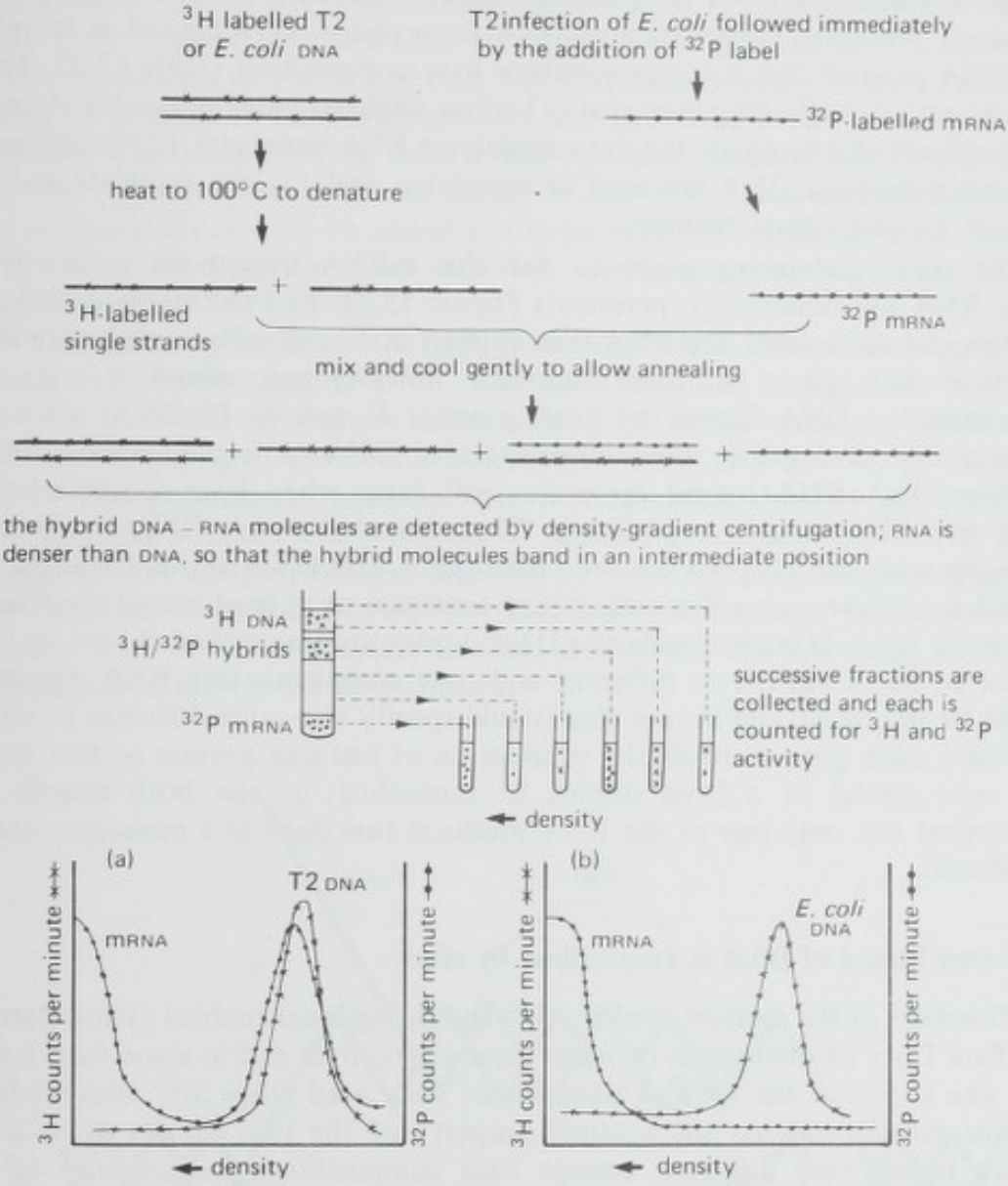


Figure 13.2 The mRNA produced when T2 infects *E. coli* is complementary to T2 DNA and not to *E. coli* DNA. ^{3}H-labelled DNA from T2 or *E. coli* is denatured by heating to 100°C and then gently cooled in the presence of ^{32}P-labelled RNA isolated from T2-infected cells of *E. coli*. In addition to single strands of DNA and mRNA the mixture will contain some DNA duplices formed by reannealing and, if the mRNA is complementary to at least one of the DNA strands, some DNA–RNA hybrid molecules. RNA is denser than DNA and when the mixture is centrifuged in a density gradient the hybrid molecules will band in an intermediate position. Successive fractions are collected by piercing the bottoms of the centrifuge tube and the ^{3}H and ^{32}P activity of each sample is measured.

The results show that when mRNA is cooled with denatured T2 DNA (a) both the ^{32}P and the ^{3}H labels are detectable in a common set of samples, but when cooled with denatured *E. coli* DNA (b) the ^{32}P and ^{3}H labels are not detected together in the same fractions. Thus the T2-induced mRNA forms hybrids with and is complementary to T2 DNA.

In this *in vitro* system the base ratios of the single-stranded RNA product and the double-stranded DNA template are always the same (with uracil replacing thymine), but when single-stranded DNA from phage X174 is used as template the RNA product has a complementary base composition (table 13.2). These experiments strongly suggested that (1) either single-stranded or double-stranded DNA can act as a template for the assembly of RNA molecules, (2) both strands of double-stranded DNA are used as templates, and (3) the template and the product are base complementary.

The most convincing evidence for the mRNA hypothesis comes from DNA-RNA hybridisation experiments (figure 13.2). In 1961 Bernard Hall and Sol Spiegelman isolated the RNA that appears in *E. coli* cells immediately after infection with phage T2 (the supposed mRNA) and mixed it with the single-stranded DNA formed by heating either *E. coli* or T2 DNA; when the mixture is cooled gently the double-stranded DNA molecules reform and in addition DNA-RNA hybrid molecules will form when base complementary DNA and RNA strands are present. They found that this RNA would only hybridise with the T2 DNA and *not* with the *E. coli* DNA so that the supposed mRNA does have a complementary base sequence to at least one of the strands of the T2 DNA. If both strands of a DNA duplex are transcribed *in vivo*, as they appear to be *in vitro*, two different and base complementary RNA molecules would be produced, and in turn these could specify two quite different proteins. However, each gene controls the production of just one protein so that either only one strand of a DNA duplex is transcribed, or else both strands are transcribed but only one of the RNA products functions as a messenger and is translated.

Only One Strand of DNA is Transcribed *in vivo*

A refinement of the method used by Hall and Spiegelman enabled Julius Marmur and Paul Doty to distinguish between these alternatives and to show that *in vivo* only one strand of the DNA is transcribed. They used phage SP8, which infects *Bacillus subtilis* and has the unusual property of the two strands of its DNA duplex having very different average base compositions, the majority of the comparatively heavy purines being localised along the same strand, the 'heavy' strand. If the DNA of SP8 is denatured by heating to 100°C the heavy purine-rich and the light pyrimidine-rich strands can be separated by density-gradient centrifugation. Marmur and Doty isolated the mRNA that appears in *B. subtilis* after infection with SP8, mixed it with either the 'light' or the 'heavy' strands of SP8 DNA and allowed the mixture to cool. They found that the SP8 mRNA *only* formed DNA-RNA hybrids with the 'heavy' DNA strand. Quite clearly only the 'heavy' strand of the SP8 DNA has served as a template for the formation of SP8 mRNA.

Synthesis is in the 5′ to 3′ Direction

When mRNA is formed against a DNA template, either *in vitro* or *in vivo*, the

RNA product is always synthesised in the 5′ to 3′ direction. This was first shown by growing *E. coli* at 0° C and feeding the cells with ^{14}C-labelled uridine. At 37° about forty nucleotides per second are added to the growing mRNA but at 0° mRNA synthesis is very slow and only one nucleotide is added every 13 seconds, making it possible to label differentially the two ends of the growing mRNA molecule. When this growing mRNA was extracted and analysed it was found that the ^{14}C label first appeared at the 3′ end of the growing molecule; since new nucleotides can only be added at the growing end, synthesis must proceed in the 5′ to 3′ direction. This direction of synthesis is also shown by the experiment described on page 22.

We are still very largely in the dark as to exactly how the mRNA is templated against the double-stranded DNA. The most favoured explanation is that a molecule of RNA polymerase moves along the DNA molecule causing the localised unwinding of the double helix (figure 13.3), and that within the limits of the RNA polymerase molecule free ribonucleoside triphosphates form base pairs with the exposed bases on *one* of the DNA strands (figure 13.4). These ribonucleotides are polymerised together by the RNA polymerase producing a short segment of hybrid DNA-RNA; eventually, the nascent mRNA molecule unwinds from its DNA template to form mRNA and the two single strands of DNA anneal to reform the DNA duplex.

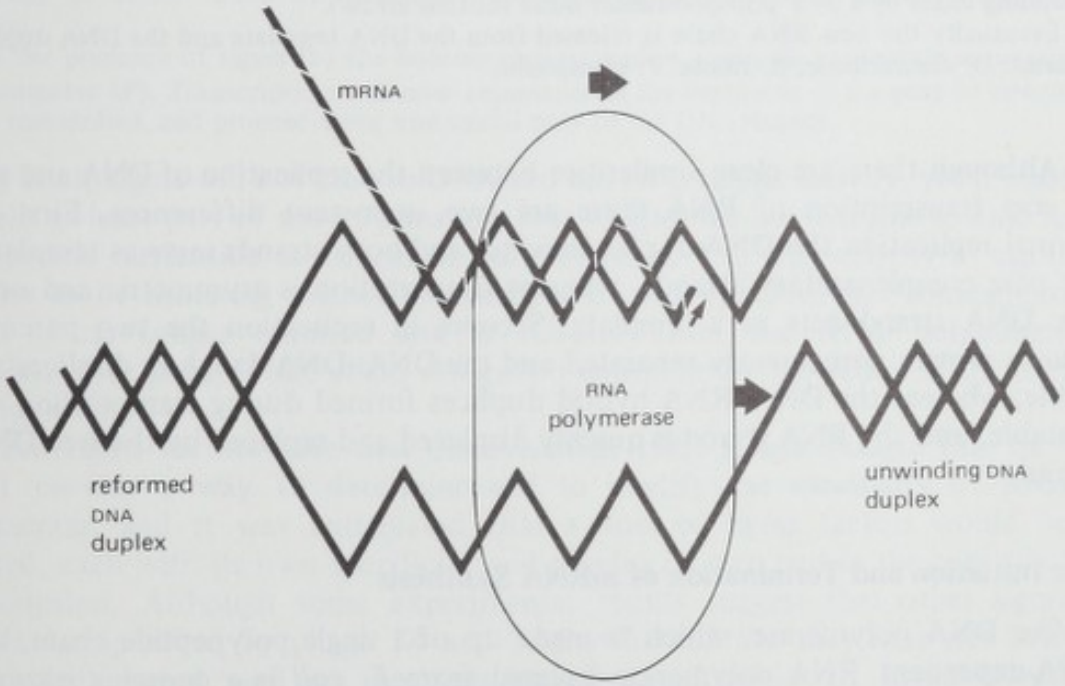


Figure 13.3 The synthesis of messenger RNA. A molecule of RNA polymerase moves along the DNA duplex, causing it to unwind. One of the single strands produced acts as a template for the production of a base-complementary strand of mRNA which then separates from the template DNA. After the RNA and DNA have separated the two strands of DNA reanneal to reform the duplex.

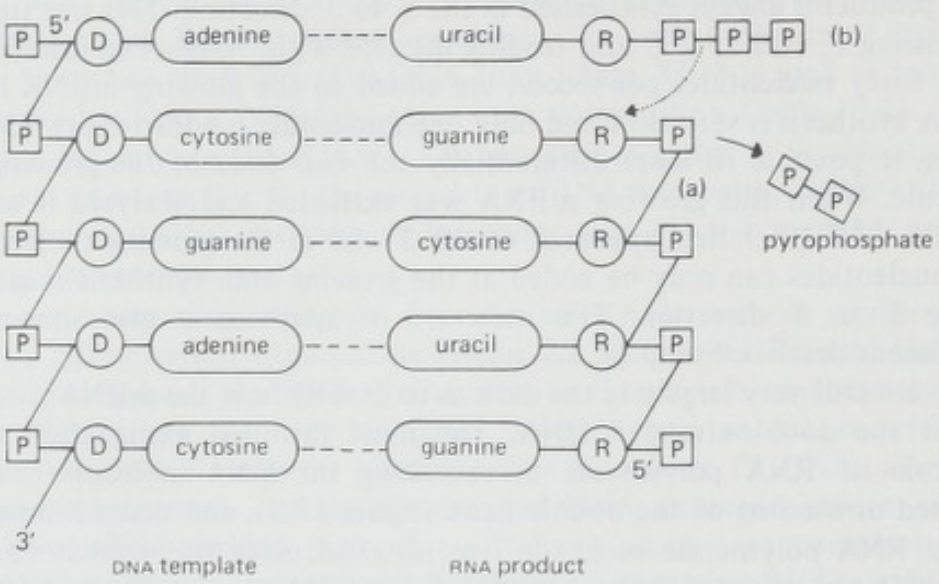


Figure 13.4 RNA is formed by a template mechanism. RNA polymerase operates by accurately templating a molecule of RNA (right) against one strand of a DNA duplex (left) in the 5′ to 3′ direction.

(a) A molecule of guanosine triphosphate (GTP) has base paired with its complementary base on the DNA template and RNA polymerase has joined it on to the 3′ end of the new RNA chain, releasing a molecule of pyrophosphate.

(b) A molecule of UTP has lined up on the template and is about to be joined on to the extending chain by a 3′:5′ phosphodiester bond (dotted arrow).

Eventually the new RNA chain is released from the DNA template and the DNA duplex reforms. D: deoxyribose; R: ribose; P: phosphate.

Although there are close similarities between the replication of DNA and the *in vivo* transcription of RNA there are two important differences. First, in normal replication the DNA duplex unwinds and both strands serve as templates for new complementary strands, whereas transcription is asymmetric and only one DNA strand acts as a template. Second, in replication the two parental strands remain permanently separated and the DNA-DNA daughter duplices are stable, whereas the DNA-RNA hybrid duplices formed during transcription are unstable, and the RNA strand is quickly displaced and replaced by the free DNA strand.

The Initiation and Termination of mRNA Synthesis

Unlike DNA polymerase, which is made up of a single polypeptide chain, the DNA-dependent RNA polymerase isolated from *E. coli* is a complex enzyme which can be dissociated into a number of component polypeptides. The complete or *holoenzyme* has a MW of about 500 000 and is composed of: two identical alpha chains (α); one beta (β) chain; one beta-prime (β') chain, similar but not identical to the β chain; and one sigma (σ) factor. It is formulated $\alpha_2\beta\beta'\sigma$. The holoenzyme without the sigma factor is known as *core* enzyme, represented $\alpha_2\beta\beta'$.

The core enzyme by itself is able to polymerise RNA against a DNA template, but not only is the reaction very inefficient but transcription is initiated from random points on both strands of the DNA duplex (figure 13.5a); it is thought that this transcription is initiated because the core enzyme makes starting mistakes. However, when sigma is added the synthetic activity of the enzyme is restored; the holoenzyme recognises specific nucleotide sequences on the DNA duplex (promoters) so that not only is the correct strand transcribed but also mRNA synthesis commences adjacent to the beginning of a gene or an operon (figure 13.5b).

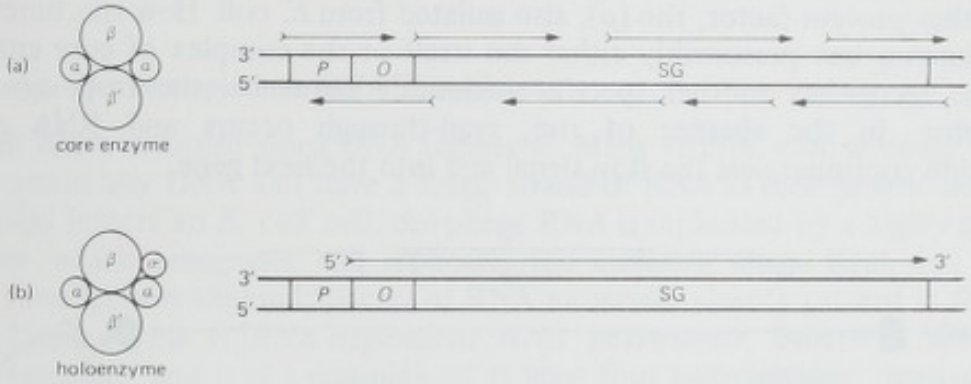


Figure 13.5 The initiation of transcription. In the absence of sigma, the core enzyme binds randomly to either strand of the DNA duplex (a) so that transcription is initiated at random.

In the presence of sigma (b) the holoenzyme recognises a specific nucleotide sequence, the promoter (*P*). Transcription will now commence at the beginning of the gene or operon to be transcribed, and proceed along one strand only of the DNA duplex.

By itself sigma will not bind to DNA and has no catalytic activity, yet it must contain at least part of the information that enables the holoenzyme to bind to the specific nucleotide sequences of the *promoters*. The only activity of sigma appears to be initiating mRNA synthesis, and once synthesis has commenced sigma is no longer required and is released from the RNA polymerase complex—thus polypeptide chain elongation continues by the action of the core enzyme alone.

When sigma factors were first discovered in 1969 it was thought that they might provide a way to determine and to modify the specificity of RNA polymerase, and it was anticipated that a host of sigma factors would be isolated, each with its own specificity and playing its own part in the control of transcription. Although some experimental results suggest that other sigma factors are specified by phage T4 and by *Bacillus subtilis*, they have not been fully substantiated, and so the question of a multiplicity of sigma factors remains open.

When the DNA of *E. coli* and of many bacteriophages is transcribed, the majority of the RNA chains produced begin with either an adenine or a guanine nucleoside triphosphate, and so the first base on the DNA to be transcribed must be a pyrimidine; it is suspected that the promoter regions contain clusters of pyrimidines on the strand to be transcribed.

Several other proteins have been identified which are involved with the *in vitro* initiation of RNA synthesis and although it may turn out that such proteins are a general requirement for transcription there is as yet no evidence to suggest that they act by initiating the transcription of specific genes. The action of one of these proteins (known as the CAP protein) in regulating the activity of certain groups of genes is described in chapter 15.

When elongation has proceeded to the end of the gene or operon there is another mechanism which causes the termination of the RNA chain and its release from the DNA template (figure 13.6). This mechanism, known to be active during the transcription of the DNA templates of certain phages, depends on another protein factor, rho (ρ), also isolated from *E. coli*. How rho functions is not known but presumably either rho itself or the complex of core enzyme and rho recognises another specific nucleotide sequence, the stop signal or *terminator*. In the absence of rho, read-through occurs and RNA chain elongation continues past the stop signal and into the next gene.

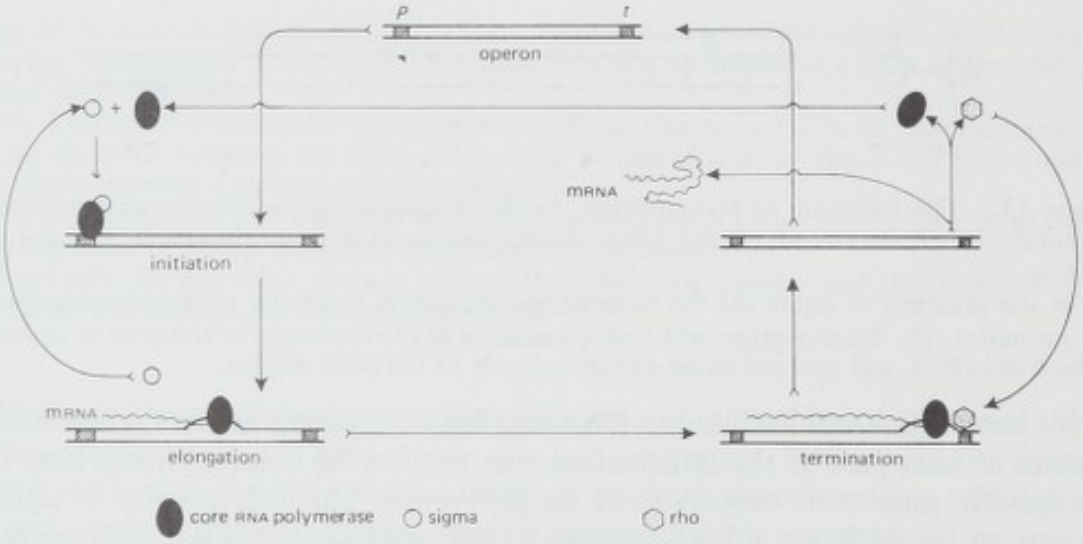


Figure 13.6 Transcription. Transcription is initiated by the core enzyme plus sigma binding to the promoter (*P*). The enzyme moves along the DNA templating a strand of nascent mRNA in the 5′ to 3′ direction, and the sigma factor is released to recycle. After the operon has been transcribed the termination factor rho causes the cessation of mRNA synthesis. The core enzyme and rho recycle, and the completed molecule of mRNA is released.

RNA can be Processed after Transcription

The immediate products of transcription are sometimes inactive and have to be modified before they can show biological activity. These modifications include the chemical alteration of the bases, the addition of one or more nucleotides, the removal of nucleotides and the cleavage of the RNA into smaller pieces. A very good example is the post-transcriptional processing of the precursor molecules of transfer RNA (the structure of tRNA is described in chapter 14). The immediate transcript of a tRNA gene is known as precursor tRNA, and before it can

become active as a molecule of transfer RNA it must be modified in at least three ways. First, in both eucaryotes and procaryotes the precursor tRNA is up to thirty nucleotides longer than the active molecule and nuclease activity must 'trim off' the excess nucleotides. Second, some 2 per cent of the bases in tRNA are methylated; this methylation is due to the activity of specific tRNA methylases acting on the bases in precursor tRNA. Third, all tRNA molecules terminate with the sequence pCpCpA at their 3′ end; these three nucleotides are added sequentially after transcription by a special enzyme system. Another good example is the post-transcriptional processing of 45S RNA into 18S and 28S ribosomal RNA (chapter 18).

RNA can also be copied on RNA Templates

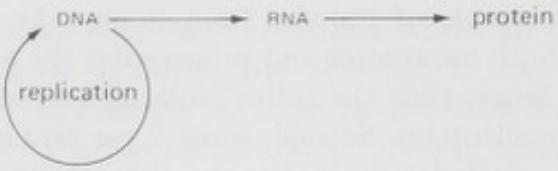
Not all RNA is copied along DNA templates. Many viruses, such as phage Qβ, do not contain any DNA and have a single strand of RNA as their genetic material. When Qβ infects an *E. coli* cell, the phage RNA is replicated by a highly specific enzyme which recognises and replicates the infecting phage RNA and at the same time ignores the multiplicity of RNA molecules already present in the host cell. This enzyme is *RNA-dependent RNA polymerase,* otherwise known as RNA replicase, and it is a complex of at least four polypeptides; three of these (α, γ and δ) are donated by the host cell and one (β) is specified by the infecting phage genome.

Immediately upon infection the single strand of Qβ RNA acts *directly* as mRNA and the gene for the RNA replicase β subunit is translated by the synthetic machinery of the host cell (the control of transcription in these phages is discussed in chapter 15). Once the replicase has been assembled the infecting (+) strand of RNA acts as a template from the synthesis of a complementary (−) strand; this (−) strand is then released from the (+) strand template and acts as a template for the synthesis of a new viral (+) strand–both the (+) and the (−) strands are synthesised in the usual 5′ to 3′ direction.

As far as we know the synthesis of RNA upon RNA templates is confined to virus-infected cells.

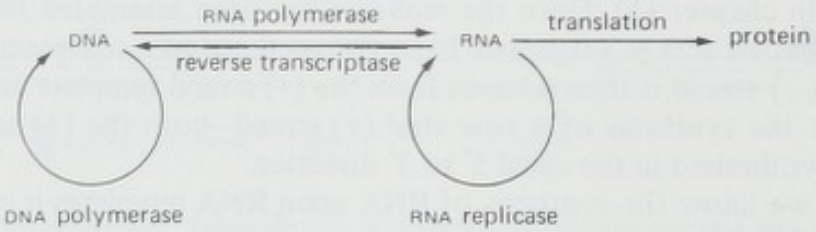
Interlude–The Central Dogma Reversed

Until very recently, the central dogma of molecular genetics was that DNA makes RNA which makes protein, and it was generally accepted that a DNA molecule could only receive its genetic information from an already existing DNA molecule. A minor modification to this dogma was required when it was shown, perhaps not unexpectedly, that RNA could replicate RNA, but it was still felt that the pathway could never operate in reserve, and so there was great excitement in 1970 when Howard Temin and David Baltimore independently discovered that certain RNA viruses control the production of an *RNA-dependent DNA polymerase,* which uses RNA as a template to make DNA; this enzyme is more generally called reverse transcriptase.



When the Rous Sarcoma Virus (RSV) infects chicken cells in tissue culture, the viral genome controls the production of reverse transcriptase (the information for this enzyme is probably encoded in the viral RNA) which then uses the single-stranded RNA as a template on which to synthesise a base complementary strand of DNA. This double-stranded DNA-RNA hybrid molecule is now replicated 'normally' by DNA polymerase to produce duplex molecules of DNA that are, in effect, copies of the viral genome. Not only can this double-stranded DNA be transcribed by the normal cellular processes to produce more single-stranded viral RNA, but also it is capable of being integrated into the continuity of the host cell DNA as *provirus*, a process broadly equivalent to the lysogenisation of a bacterium by a temperate phage (chapter 9). There is now very good evidence that certain tumour viruses, such as RSV and SV40, do become a part of the host genome in this way and that in doing so they induce changes in the phenotype of the host cell.

In view of this discovery the central dogma must be restated as 'nucleic acid makes protein' and so far there is no evidence to suggest that the reverse is ever true. These relationships between DNA, RNA and protein can be summarised diagrammatically as follows:



Summary of Key Points

(1) RNA is directly involved in protein synthesis.
(2) Proteins are assembled on the ribosomes.
(3) The first step in protein synthesis is transcription, the formation of a single strand of RNA base complementary to *one* of the two strands of DNA being transcribed. This is messenger RNA or mRNA.
(4) mRNA is synthesised in the 5′ to 3′ direction by templating against DNA. Synthesis starts with RNA polymerase binding to a specific nucleotide sequence, the promoter, adjacent to the beginning of the genes to be transcribed.

(5) Specific protein factors assist in the initiation and termination of mRNA synthesis.
(6) Cellular RNA, such as ribosomal RNA and transfer RNA, is transcribed by the same process.
(7) In virus-infected cells, RNA can act as a template for its own replication.
(8) Although the central dogma of molecular genetics was that DNA makes RNA, which makes protein, recent studies show that in tissue culture cells infected by RNA tumour viruses the RNA acts as a template for the synthesis of a base complementary strand of DNA—a reversal of the central dogma.
(9) Special technique described is the *in vitro* synthesis of RNA.

14 Protein Synthesis: Translation

This suspense is terrible. I hope it will last.
Oscar Wilde (1854–1900)

In the last chapter we saw how the first step in protein synthesis is the formation of a single-stranded messenger RNA complementary to one of the strands of DNA. We now have to consider how the four-letter nucleic acid language of the genetic code on the mRNA is *translated* into the twenty-letter amino-acid language of polypeptides. When mRNA is templated against DNA, the genetic information, encoded in the base sequence, is maintained because of the specific hydrogen bonding that occurs between pairs of nucleotides; however, the amino acids do not have any such specific affinity with the purine and pyrimidine bases of mRNA so that an adaptor is necessary which will recognise the nucleotide sequence of a specific mRNA codon on the one hand, and a particular amino acid on the other. The existence of these adaptors, or transfer RNA molecules as we now call them, was first postulated by Crick in 1957 and within a year their existence had been confirmed by Mahlon Hoagland and his co-workers. A third class of RNA is also required for protein synthesis, the ribosomal RNA; this is the main constituent of the ribosomes, the actual sites of protein synthesis (page 217). Like mRNA, both tRNA and rRNA are transcripts of chromosomal DNA, but unlike mRNA they do not serve as templates for the assembly of proteins.

The Ribosomes

The ribosomal RNA (rRNA) of *E. coli* constitutes 75–85 per cent of the total cellular RNA and is associated with the ribosomal proteins (about 40 per cent by weight) to form the ribosomes. The ribosomes constitute about 25 per cent of the cell mass; each is a small spherical particle about 20 nm in diameter. Each 70*S* ribosome is composed of two subunits which reversibly dissociate at low Mg^{2+} concentrations. (*S*, the *sedimentation coefficient*, measures the rate of sedimentation of a particle when ultracentrifuged at high speed, and is directly proportional to the size of the particle.) The larger 50*S* subunit (figure 14.1) contains a molecule each of 5*S* and 23*S* rRNA and about thirty-four ribosomal proteins, while the smaller 30*S* subunit contains a molecule of 16*S* rRNA and about twenty specific proteins. Most of the thirty-four or so ribosomal proteins are present as one molecule per ribosomal subunit, suggesting that each subunit is a unique complex of ribosomal RNAs and proteins. We do not fully understand the functions of these components, but some of the proteins are the 'factors' required for translation (page 259).

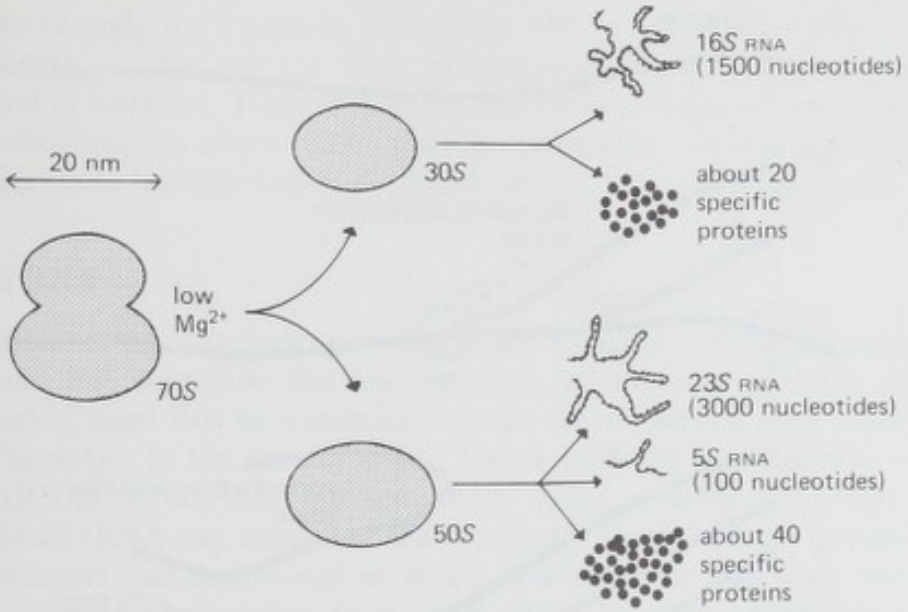


Figure 14.1 The components of a ribosome. At Mg^{2+} concentrations below 0.01M the 70*S* ribosome breaks down into 30*S* and 50*S* sub-units. In turn, these consist of ribosomal RNA and specific proteins. The ribosomes of higher organisms are similar but may have higher *S* values.

The ribosomal RNA contains non-equivalent amounts of A and T and of C and G (table 13.1), and although the molecules are single-stranded there are extensive double helical regions where the bases are hydrogen bonded together in hairpin-like turns (figure 14.2).

Messenger RNA molecules are the templates for the assembly of specific proteins, and a polypeptide is synthesised by a ribosome attaching to the mRNA, moving along it in the 5′ to 3′ direction, and as it does so adding successive amino acids to the growing polypeptide chain (figure 14.3) in a sequence determined by the nucleotide sequence of the mRNA. In other words, the ribosome reads the genetic message of each mRNA codon and selects the appropriate amino acid for addition to the elongating polypeptide chain. When

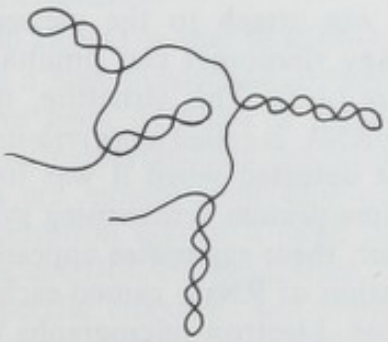


Figure 14.2 Ribosomal RNA. Although single-stranded, parts of the rRNA molecules are in the form of hairpin-like loops with the opposite bases H-bonded together.

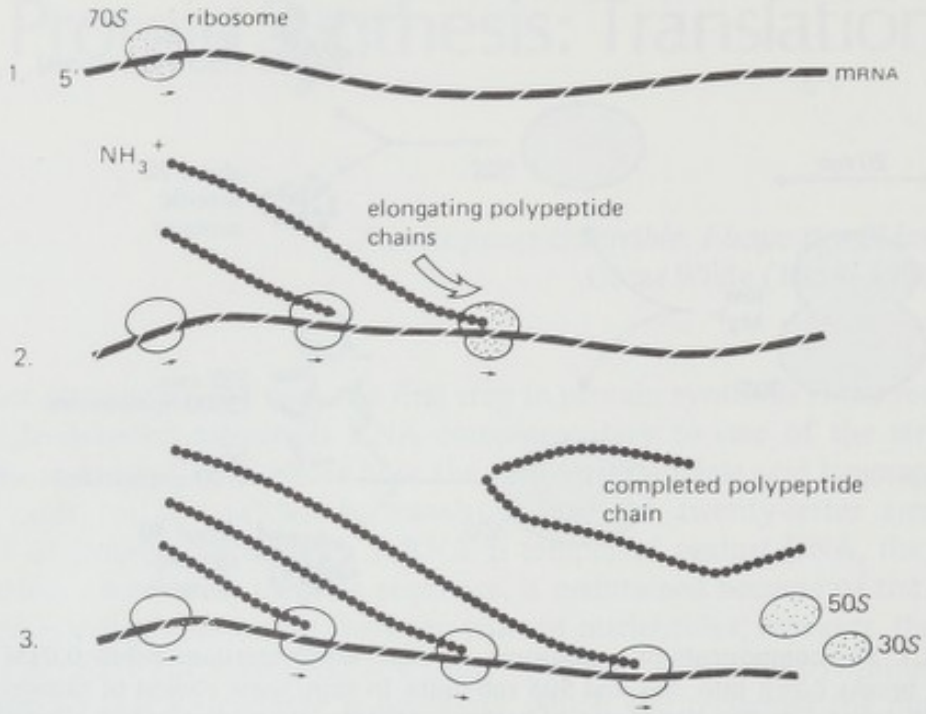


Figure 14.3 Translation. A ribosome attaches to the 5′ end of the tRNA molecule (1), and as it moves along the messenger a nascent polypeptide chain is synthesised. Another ribosome can now attach to the mRNA (2), and it also will produce a polypeptide as it moves along the messenger. This process is repeated, and there may be one ribosome for every eighty nucleotides along the messenger (3). The mRNA with all the attached ribosomes is a *polyribosome.*

the ribosome reaches the end of the genetic message, the polypeptide is completed and the ribosome dissociates, is released and recycles.

Polyribosomes

However, polypeptide synthesis does not involve a single ribosome working with the molecule of mRNA but the coordinated action of several ribosomes working in unison. After a ribosome has attached to and started to move along the mRNA, another ribosome can attach to the messenger and also commence translation; in this way many ribosomes can simultaneously translate a single molecule of mRNA (figure 14.3). This structure, of several ribosomes held together by a molecule of mRNA, is called a *polyribosome* or *polysome.*

Polyribosomes were first detected when it was found that the addition of synthetic mRNA to a cell-free protein synthesising system of *E. coli* caused the ribosomes to clump together; these aggregates appeared to be held together by RNA since a low concentration of RNase caused each polyribosome to separate into several single ribosomes. Electron micrographs of ribosomes from rabbit reticulocytes synthesising haemoglobin showed the ribosomes in clusters of four to six, held together by a thin thread about 150 nm long and about 1–1.5 nm in diameter. This thread is most probably mRNA, and if the distance between

adjacent nucleotides is 0.35 nm it would be about 430 nucleotides long, sufficient to code for a protein containing 140 to 145 amino acids—almost the exact length of one of the four polypeptide chains which makes up the haemoglobin tetramer. There was an average of five ribosomes per polyribosome, or one ribosome for every eighty mRNA nucleotides, so that very large mRNA molecules could be simultaneously translated by up to fifty ribosomes.

Transfer RNA

The ribosomes are the factory of the living cell, and the mRNA molecules the blueprints, but somehow the raw materials for protein synthesis, the twenty amino acids, must first be transported to the ribosomes and then lined up on the mRNA template in the correct order. This is achieved by molecules of transfer RNA (tRNA) or, as they are sometimes called, soluble RNA (sRNA). Each molecule of tRNA can recognise and covalently bond with one particular amino acid, transport that amino-acid molecule to a ribosome and there recognise the mRNA codon corresponding to its own amino acid. In 1956 Crick postulated the existence of such adaptor molecules and went so far as to suggest that they might contain nucleotides and so be able to join on to the mRNA template by the formation of hydrogen bonds between complementary base pairs. In 1957 Paul Zamecnik's group isolated a new type of RNA from rat liver cells which turned out to be a *stable* cell constituent but was not a structural component of the ribosomes. This 'soluble' RNA fraction remained in the supernatant after disrupted cells were ultracentrifuged so as to sediment the ribosomes, and it had the unique property of binding to the amino acids in the presence of ATP. Further studies soon revealed that the 'soluble' RNA fraction contained many different species of RNA each of which was able to bind exclusively to one particular amino acid; these were clearly the adaptor molecules postulated by Crick the previous year.

Transfer RNAs are short molecules only 76–87 nucleotides long (MW 25000–30000) and in *E. coli* they constitute about 15 per cent of the total bacterial RNA. The base composition of tRNA from different sources is remarkably constant and in *in vitro* systems the tRNAs from these varied sources are interchangeable, strengthening still further the argument that the same genetic code is used by all organisms.

A striking feature of all tRNAs is that in addition to the four usual bases (A, U, C and G) they contain a high proportion of unusual bases (table 14.1). Over thirty unusual bases have been detected in tRNA, but four, dihydrouracil, pseudouracil, thymine and dimethylguanine, are the most common. It is unlikely that these unusual bases are incorporated into a tRNA molecule when it is initially formed by transcription against a DNA template; it is probable that ordinary bases in the nascent tRNA molecule are changed *in situ* into unusual bases by the action of specific enzymes, and some of the enzymes which can effect these modifications have already been isolated (see page 227).

One outstanding achievement of nucleic acid research was the determination of the complete nucleotide sequence of a molecule of alanine transfer RNA,

Table 14.1 The nucleoside compositions of some transfer RNAs

		Transfer RNA			
		alanine (yeast)	methionine (*E. coli*)	valine (*E. coli* B)	valine (yeast)
(A) *The 'usual' nucleosides*					
adenosine		8	18	14	14
cytidine		23	19	23	20
guanosine		25	18	23	19
uridine		11	10	9	11
(B) *The 'unusual' nucleosides*					
l-methyladenosine	(A)			1	1
adenosine derivative	(A)		1		
cytidine derivative	(C)		1		
5-methylcytosine	(C)				1
l-methylguanosine	(G)	1			1
dimethylguanosine	(G)	1			
7-methylguanosine	(G)		1	1	
2′-O-methylguanosine	(G)		1		
inosine	(G)	1			1
l-methylinosine	(G)	1			
ribosylthymine	(U)	1	1	1	1
5,6-dihydrouridine	(U)	2	3	1	4
4-thiouridine	(U)		1	1	
pseudouridine	(U)	2	2	1	4
uridine derivative	(U)	1		1	
unidentified			1		
Total nucleosides		77	77	76	77
Unusual nucleosides		10	12	7	13

The unusual nucleosides pair as if they were the nucleosides indicated in parentheses.

reported by Robert Holley and his colleagues in late 1965. This task took seven years to complete, four of these years being spent on the isolation from yeast of 1 gram of pure alanine tRNA—a remarkable example of painstaking and methodical research.

The primary structure of this transfer RNA is a sequence of seventy-seven ribonucleotides, and although there are no long complementary base sequences this single-stranded ribopolynucleotide is almost certainly looped back on itself several times so as to form the maximum number of guanine-cytosine base pairs. The three hydrogen bonds between guanine and cytosine are stronger than the two between adenine and thymine and so ensure that the molecule has a stable secondary structure. This is the so-called clover-leaf structure, with four

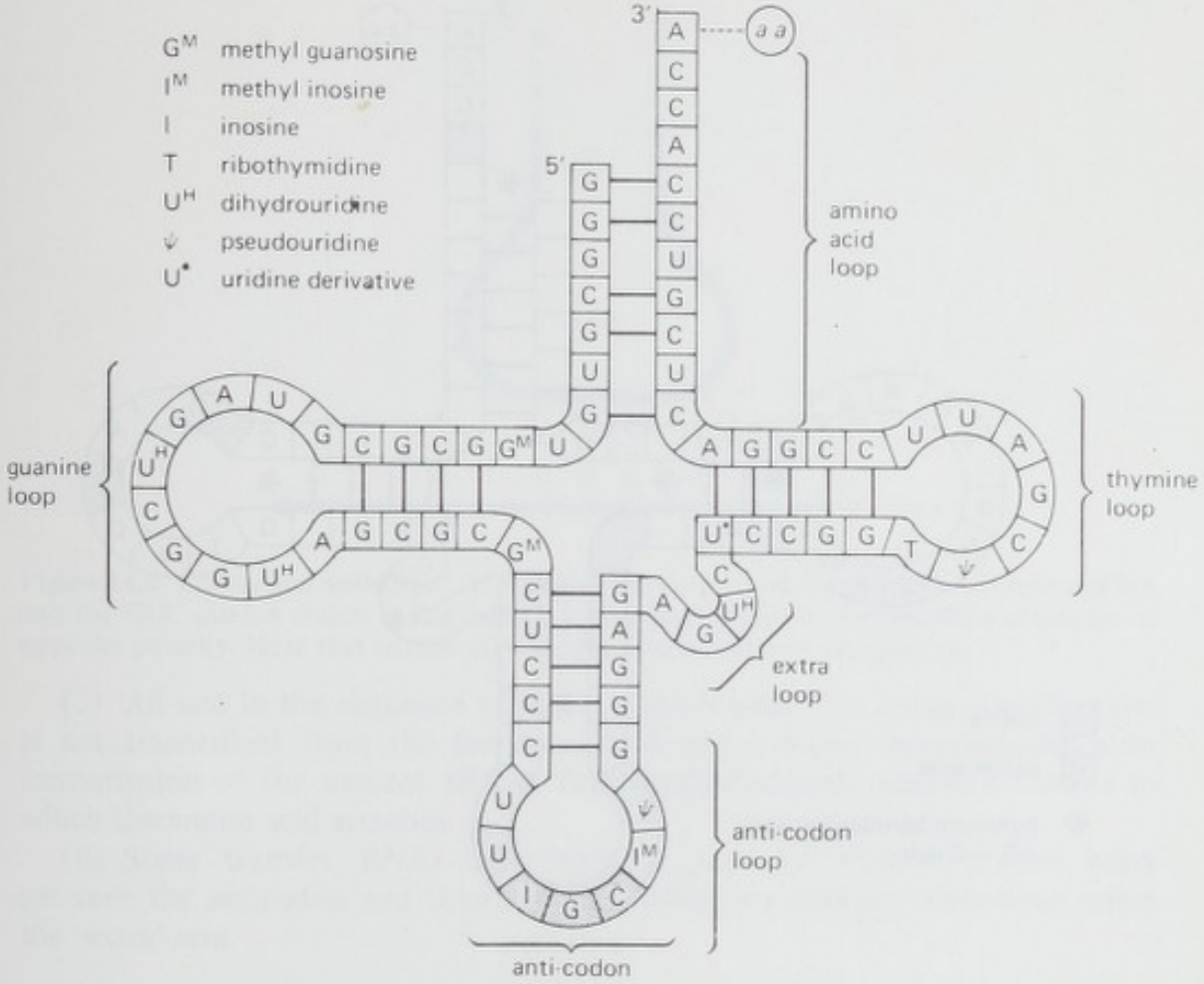


Figure 14.4 The primary and secondary structure of alanine-tRNA from yeast. This tRNA is seventy-seven nucleotides long and the molecule is folded back on itself several times so as to form the maximum number of G–C base pairs—the four double-stranded regions hold the molecule together in the so-called 'clover-leaf' structure.

double-stranded regions (figure 14.4), and this in turn is probably elaborately folded into a tertiary structure. It will be noted that the 'unusual' bases, many of which are unable to form base pairs by hydrogen bonding, are located either in the bends or in the unpaired ends of the hairpin loops, and it is thought that one of the functions of these bases is to prevent the formation of hydrogen-bonded base pairs so leaving exposed the 'reactive' parts of the molecule.

Since 1966 the primary structures have been determined of more than ten transfer RNAs from organisms as diverse as *E. coli*, wheat and rats, and all have base sequences which allow them to be folded into similar clover-leaf shaped structures. These transfer RNAs all form regular three-dimensional crystals and so probably also have similar tertiary structures.

tRNA—Structural Homologies and Functions

All these transfer RNAs have a number of common features, summarised in

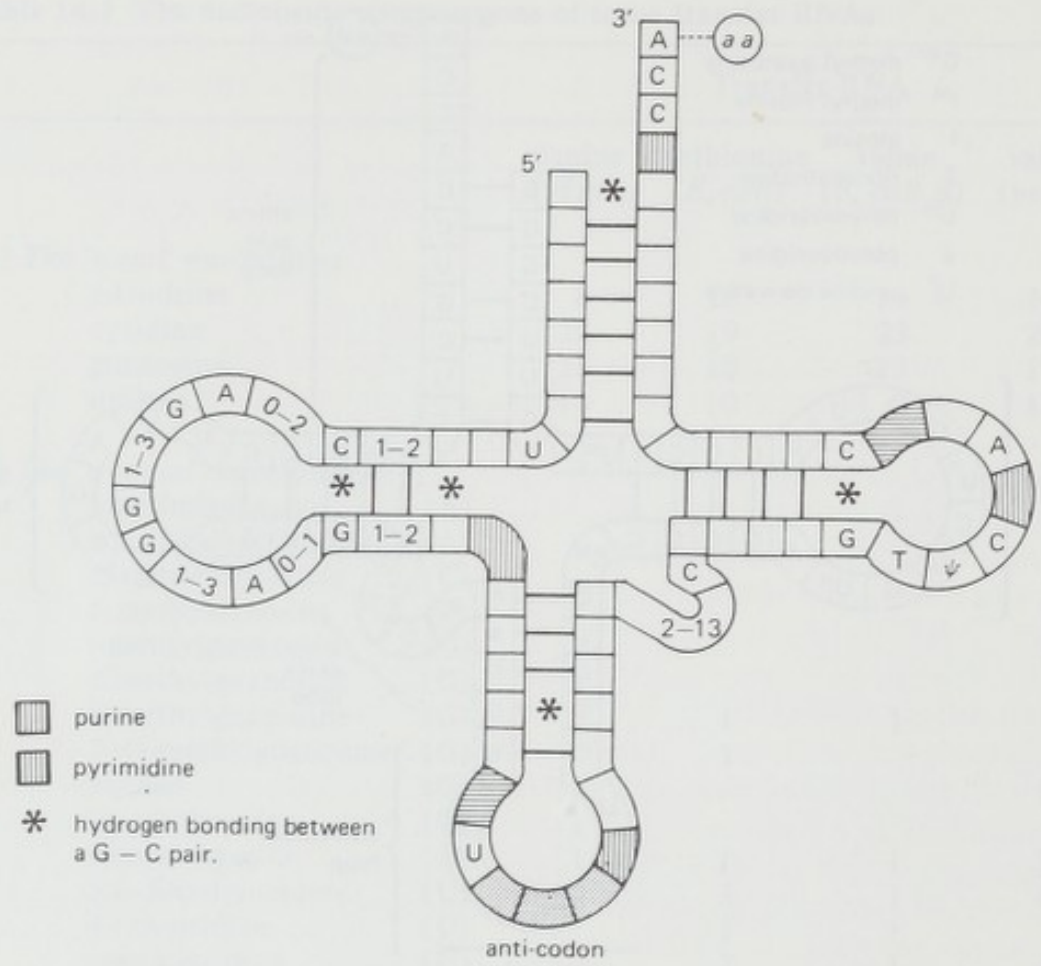


Figure 14.5 Common features of transfer RNA molecules.

figure 14.5. Proceeding around the molecule from the 5′ terminal, note that

(1) All terminate at the 5′ terminal with either Gp (most) or Cp.

(2) All have a guanine-rich arm containing three or four pairs of bases and a loop of eight to twelve exposed (that is, unpaired) bases. The function of this arm is not yet clear.

(3) All have an anticodon arm containing five pairs of bases and a loop of seven exposed bases. Each tRNA molecule is able to recognise a specific codon on the messenger RNA, and the simplest way it can do this is by carrying a sequence of nucleotides complementary to the sequence of nucleotides in one of the corresponding messenger RNA codons; such a sequence is called an *anticodon*. The anticodon arm of each tRNA carries the sequence 5′-uracil-anticodon-purine-3′, and in relation to the codon the anticodon is always back to front; it is apparent that when the tRNA lines up on and attaches to the mRNA codon the pairing is 'antiparallel'—that is, the two ribopolynucleotide strands are polarised in opposite directions (figure 14.6).

(4) All have a thymine arm containing five pairs of bases and a loop of seven unpaired bases containing the sequences 5′-T-ψ-C-G-A-3′ or 5′-T-ψ-C-A-A-3′.

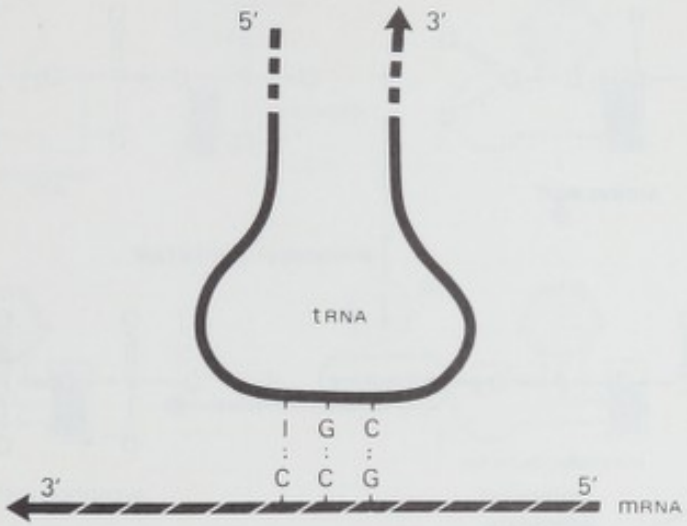


Figure 14.6 Codon and anticodon. The pairing between the IGC anticodon in alanine tRNA and the GCC alanine codon in the mRNA is antiparallel; that is, the two RNA chains are of opposite polarity. Note that inosine (I) is derived from, and pairs as, guanine.

(5) All end in the sequence pCpCpA at the 3′ end. This group of nucleotides is not transcribed from the template DNA but is added enzymatically after transcription of the nascent tRNA. The terminal adenine residue is the site to which the amino acid attaches.

(6) Some transfer RNAs have between two and thirteen further bases between the anticodon and thymine arms, forming a fifth arm sometimes called the 'extra' arm.

Amino-acid Activation

Before transfer RNA can act as an adaptor and transport amino acids to the ribosomes, each molecule of tRNA must attach to a molecule of its own amino acid; the tRNA is now said to be *charged* and is known as *aminoacyl-tRNA*. Thus alanine tRNA ($tRNA^{Ala}$) is charged by uniting with an alanine molecule to form alanyl-tRNA (Ala-$tRNA^{Ala}$ or Ala-tRNA). This union is catalysed by a group of specific amino acid-activating enzymes known as *amino-acyl-tRNA synthetases*. These enzymes are highly specific and a different enzyme is responsible for coupling each different amino acid with its appropriate tRNA; thus there are at least as many aminoacyl synthetases as there are amino acids.

The first step in this reaction is the activation of the amino-acid molecule by its carboxyl (-COOH) group reacting with adenosine triphosphate (ATP) to form aminoacyl adenylate and pyrophosphate (figure 14.7). Next, the aminoacyl adenylate reacts with the 3′ terminal adenyl residue on the correct tRNA, forming aminoacyl-tRNA and adenosine monophosphate (AMP); thus the amino acid has been covalently bonded to its transfer RNA by a high energy bond. These reactions occur sequentially on the specific aminoacyl synthetase (figure 14.8) and it is clear that each synthetase must have two binding sites, one recognising the specific side group (R) of an amino acid and the other recognising the corresponding tRNA molecule.

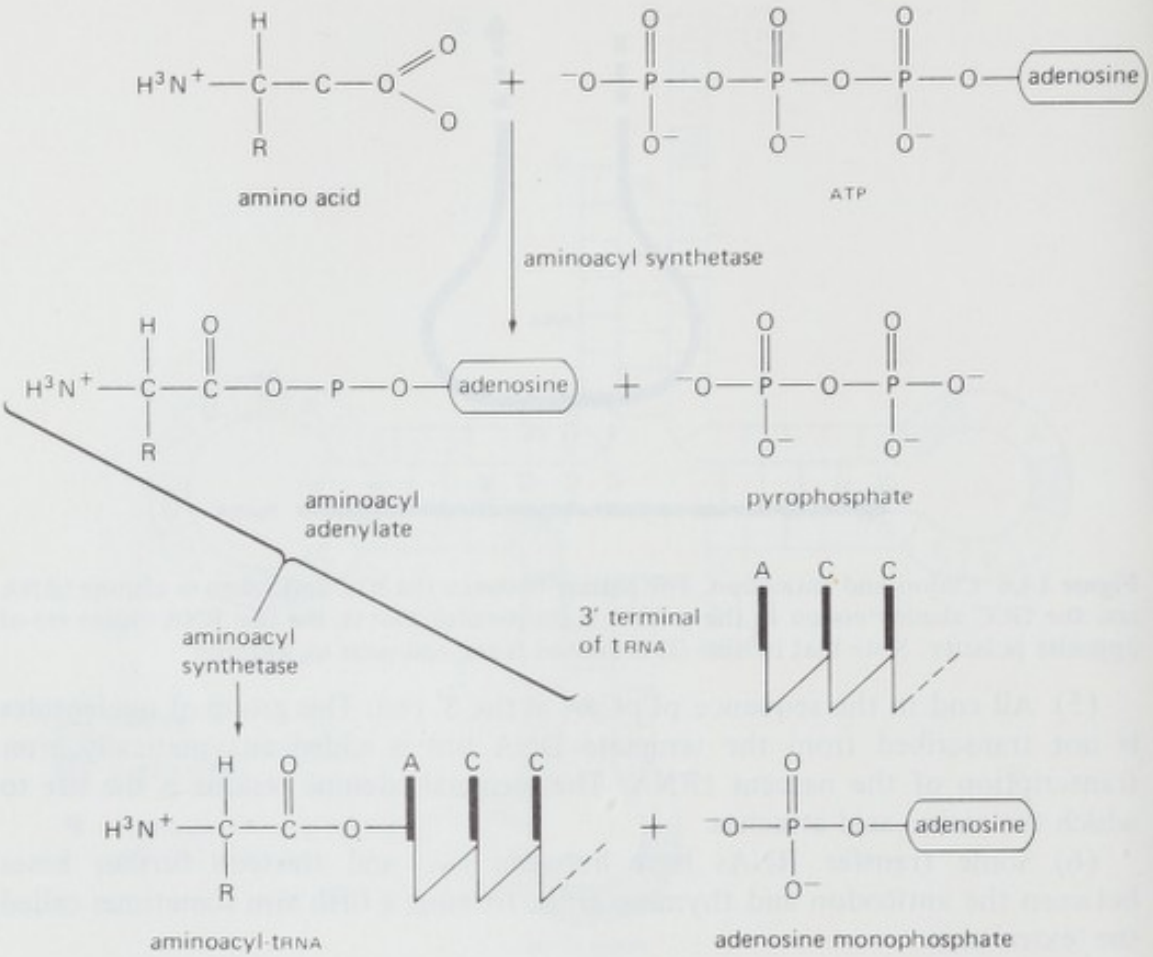


Figure 14.7 The formation of aminoacyl-tRNA (i). The amino acid is first activated by combining with ATP to form aminoacyl adenylate and then transferred from aminoacyl adenylate to the 3′ CCA terminal of a specific tRNA molecule.

We do not know just how the synthetase is recognised by its tRNA molecule. In *E. coli* the tryptophan-accepting tRNA has the anticodon CCA, and when this is changed by mutation to CUA the tRNA is chargeable with glutamine, a clear indication that the anticodon is involved. On the other hand there is evidence to suggest that the anticodon of other tRNAs is not directly involved in synthetase recognition: for example, in *E. coli* there are two different leucine-accepting tRNAs which have different anticodons, recognise different codons on the messenger RNA and yet are charged by the same aminoacyl synthetase; furthermore, mutations that change the nucleotide sequence in the amino-acid loop of a tyrosine-accepting tRNA enable this tRNA to be charged with glutamine. It seems clear that there is no single recognition site, and it may well be that recognition is dependent on the three-dimensional configuration of the tRNA molecule rather than on specific base sequences.

The messenger RNA, the transfer RNA molecules, the amino acids, the aminoacyl synthetases and the ribosomes are the principal components of the extremely complex machinery for protein synthesis, and it remains to see how these act together to produce specific polypeptides. This process of translation is

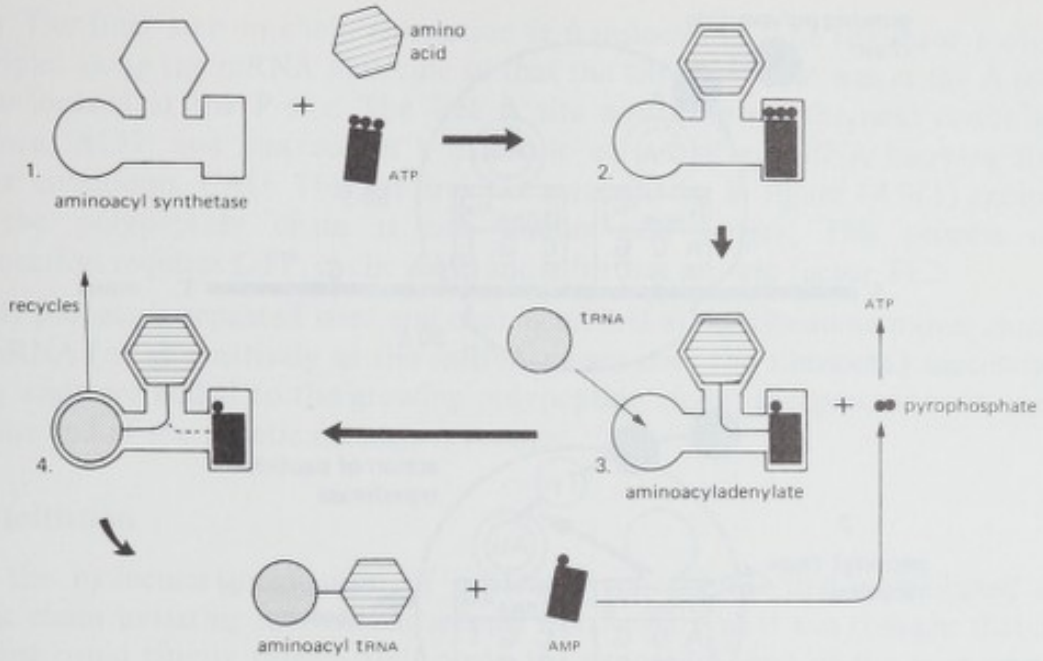


Figure 14.8 The formation of aminoacyl-tRNA (ii).

1. The enzyme aminoacyl synthetase acts as a template to which the separate components can bind and react together.

2. The amino acid and ATP bind to the enzyme and react to form aminoacyl adenylate and pyrophosphate.

3. A molecule of specific tRNA binds to the enzyme and the bond between the amino acid and AMP is transferred to the molecule of tRNA.

4. Finally, the aminoacyl-tRNA and AMP are released from the enzyme, which now recycles.

most simply understood by considering (1) chain elongation–how is it that successive amino acids are added to the end of the growing polypeptide chain? (2) Chain initiation–what is the specific mechanism that tells the translational machinery to 'start here' at the beginning of a message encoded in a molecule of mRNA? (3) Chain termination–how does the ribosome know when it has reached the end of a message in mRNA and so terminate chain elongation?

Chain Elongation

The ribosomes translate into protein *any* messenger RNA they encounter by orientating the aminoacyl-tRNA molecules so that the code is accurately read. They can do this because they contain a specific surface to bind a molecule of RNA and two surfaces which specifically bind tRNA, so bringing all the molecules into correct alignment. The first of the tRNA binding sites is the *aminoacyl* (A) site; molecules of free aminoacyl-tRNA can attach to this site provided that (i) the anticodon on the tRNA matches the codon on the mRNA that is displayed there, and (ii) the aminoacyl tRNA is complexed with guanosine triphosphate (GTP) and a specific protein elongation factor known as TF1 (or Tu). The second site is the *peptidyl* (P) site; this will not bind free

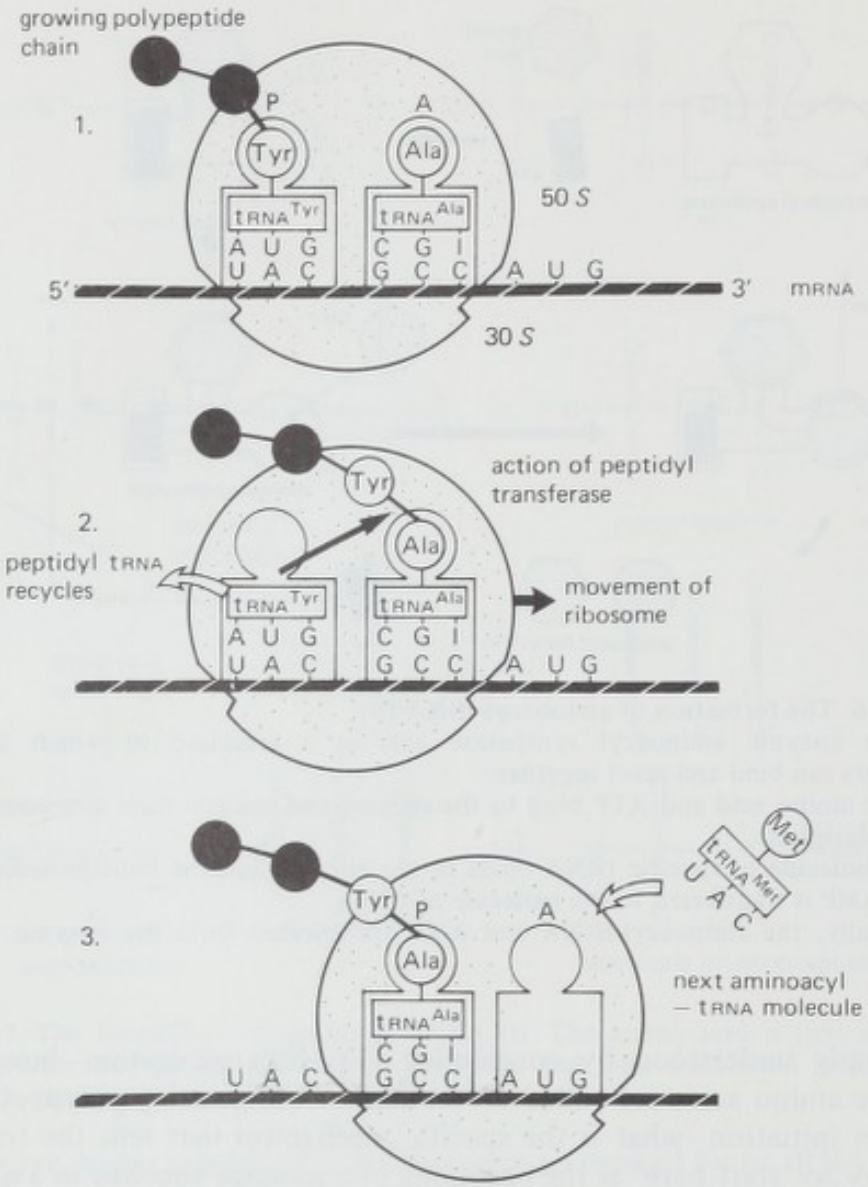


Figure 14.9 Polypeptide chain elongation. This figure is explained in the text.

tRNA and will only accept tRNA molecules from the aminoacyl site. The process of chain elongation is summarised in figure 14.9.

(1) The elongating polypeptide chain is joined by its carboxyl end to a molecule of $tRNA^{Tyr}$ located at the P site and a molecule of aminoacyl-tRNA has attached to the A site; because the next codon to be translated is 5′ GCG 3′, Ala-tRNA with the anticodon 5′ IGC 3′ has been bound.

(2) An enzyme transfers the carboxy terminal of the growing peptide chain from the $tRNA^{Tyr}$ at the P site to the amino group of the newly accepted alanyl-tRNA at the A site. The enzyme catalysing the formation of this bond, peptidyl transferase, is one of the proteins of the 50 *S* subunit. This transfer releases the molecule of $tRNA^{Tyr}$ from the P site.

(3) The final step in chain elongation is translocation. The ribosome moves one triplet along the mRNA molecule so that the $tRNA^{Ala}$ that was at the A site is now located at the P site. The free A site now displays the next codon in sequence, AUG, and can accept a molecule of methionyl-tRNA carrying the correct anticodon, CAU. This restores the state shown in figure 14.9(1) except that the polypeptide chain is one amino acid longer. This process of translocation requires GTP, cyclic AMP and a further protein factor TF2.

This process is repeated over and over again and as the ribosome moves along the mRNA (or alternatively as the mRNA passes over the ribosome) successive amino acids are added to the growing polypeptide chain; this process continues until the end of the genetic message is reached.

Chain Initiation

When the molecular mechanism of protein synthesis was first investigated a specific chain initiating mechanism seemed unnecessary as it was thought that a ribosome could simply thread itself on to the exposed 5′ end of the messenger RNA and commence translation. It was soon realised that this explanation was too simple as, first, the message could easily be read out of phase; second, many messengers are more than one gene long and it should be possible to initiate translation at the beginning of each gene along the message; and third, it is possible to translate directly single-stranded *circular* DNA where there is no free end to which a ribosome can attach.

The first clue to a specific initiation mechanism was the discovery by Frederick Sanger and his colleagues in 1967, that in *E. coli* and yeast there are two very different types of methionine-carrying tRNA, known as methionyl-tRNA (Met-$tRNA_m$) and N-formylmethionyl-tRNA (fMet-$tRNA_f$). Met-$tRNA_m$ carries a normal methionine residue at the 3′ terminus but fMet-$tRNA_f$ carries a formylated derivative of methionine known as N-formylmethionine. N-formylmethionine does not exist in the cell as such, as fMet-$tRNA_f$ is formed, firstly, by aminoacyl synthetase attaching a methionine residue to $tRNA_f$ and, secondly, by the enzymatic addition of a formyl group (O = C – H) to the amino group of the methionine. For two reasons this formylated methionyl-tRNA cannot participate in chain elongation: first, fMet-RNA_f cannot complex with GTP and the elongation factor TF2; and second, the amino group of the methionine is blocked by the attached formyl group and so *cannot* be joined onto an existing polypeptide chain. Consequently, if fMet-$tRNA_f$ is involved in protein synthesis, its only possible use is in chain initiation. No other formylated amino acid has ever been detected *in vivo*.

Surprisingly, however, when proteins isolated from *E. coli* were examined, only about 45 per cent had an N-terminal methionine and almost none had a terminal N-formylmethionine. This apparent paradox was only resolved when experiments carried out in the laboratories of Norton Zinder and James Watson showed that the formyl groups were removed from the polypeptides *after* translation. Using the very simple RNA coliphage R17 they found that the coat protein produced *in vivo* when R17 infects *E. coli* begins with the sequence

alanine - serine - asparagine - . . .

at the amino terminus, but that when R17 RNA was used as a template in an *in vitro* protein synthesising system the product commenced with the sequence

N-formylmethionine - alanine - serine - asparagine - . . .

We now know that the cells of *E. coli* contain at least two enzymes, one which can remove the formyl group from N-formylmethionine (a deformylase) leaving a terminal methionine, and another which can remove a terminal methionine from some proteins (as with the R17 coat protein) but not from others.

$tRNA_f$ and $tRNA_m$ have almost identical primary structures, including a common CAU anticodon, and we may ask how it is that an AUG codon at the start of a message is recognised only by fMet-$tRNA_f$ whereas internal AUG codons are recognised only by Met-$tRNA_m$. To put the question another way: how do fMet-$tRNA_f$ and Met-$tRNA_m$ distinguish between ribosomes located at AUG initiation codons and ribosomes located at internal AUG codons? An important point to note is that intact 70 *S* ribosomes do not bind to mRNA and

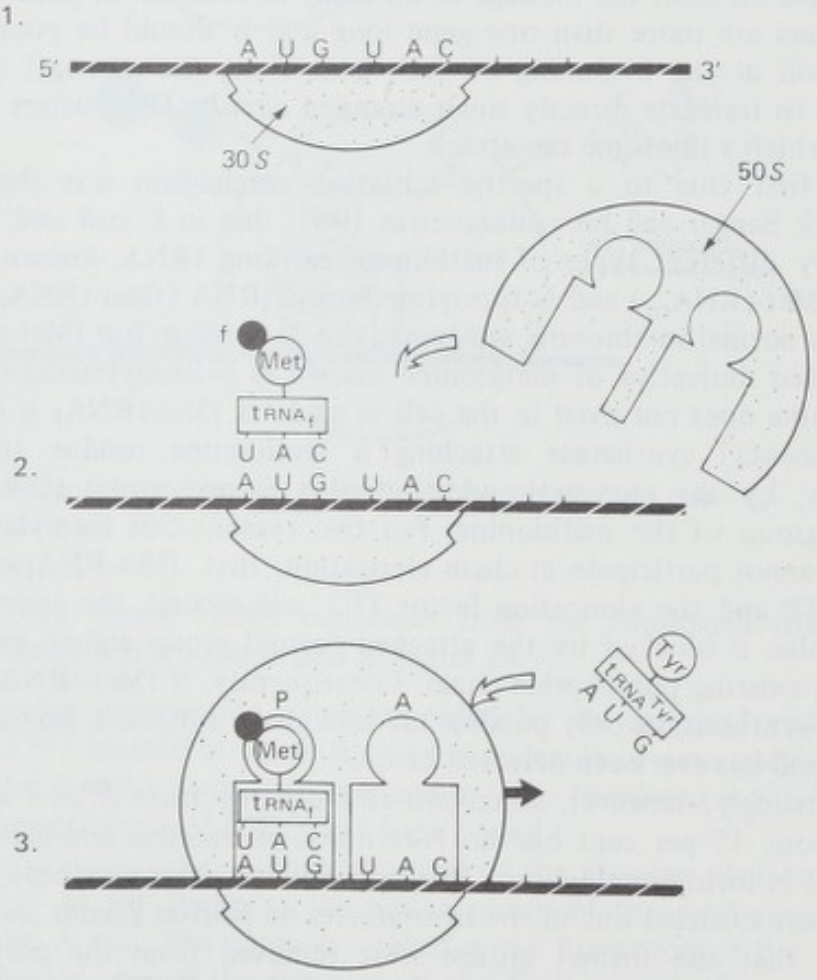


Figure 14.10 Polypeptide chain initiation. This figure is explained in the text.

that the first step in translation is the binding of a 30 *S* ribosomal subunit to the messenger, followed by the addition of a 50 *S* subunit to complete the 70 *S* ribosome—consequently, the AUG initiation codons could be translated by the 30 *S* subunits alone while the internal AUG codons could *only* be translated by the intact 70 *S* ribosomes. If this is so then initiation can be explained by the sequence of events shown in figure 14.10.

1. A 30 *A* ribosomal subunit binds to a recognition sequence on the mRNA that is adjacent to, or includes, the AUG initiation triplet and displays this codon within the 30 *S*-mRNA initiation complex.
2. fMet-tRNA$_f$ enters the initiation complex, recognises the AUG codon and locates at the P site; it is not known whether this occurs directly or via the A site. A 50 *S* subunit now attaches and completes the 70 *S* ribosome.
3. The next aminoacyl-tRNA in sequence locates at the A site and chain elongation proceeds as already described, subsequent (internal) AUG codons being recognised only by Met-tRNA$_m$.

The specificity is explained if it is assumed that fMet-tRNA$_f$ is unable to enter the intact 70 *S* ribosome-mRNA complex and that Met-tRNA$_m$ (or any other non-formylated aminoacyl tRNA) cannot enter the 30 *S* ribosome-mRNA initiation complex; thus the initiation AUG must be translated by fMet-tRNA$_f$ and internal AUG codons by Met-tRNA$_m$.

The foregoing explanation assumes that the 30 *S* ribosomal subunits bind specifically to AUG initiation and not to internal AUG triplets. Why should this be so? We do not know the answer to this question but one explanation is that the messenger RNA assumes a secondary structure in such a way that the internal AUG codons are protected by being in double helical regions (see page 249), while initiating AUG codons are in single-stranded regions and so accessible for ribosomal binding; once the ribosome has been bound it could open up the double helical regions as it moves along the messenger during translation. The difficulty with this idea is that if only a single accessible AUG triplet is required to promote specific ribosome binding, then *all* internal AUG sequences, in and out of phase, would have to be protected if random binding and translation is to be prevented. Another and more probable explanation, which largely overcomes this difficulty, is that the ribosome recognises not just an AUG codon but a specific nucleotide sequence in the region immediately preceding it; that is to say, there are specific ribosome binding sites on the mRNA. The sites on the mRNA to which ribosomes bind have been isolated by allowing free ribosomes to bind to single-stranded RNA from phages such as R17 and Qβ or to the single-stranded DNA of ϕX174 and then digesting away with nucleases all the RNA or DNA that is not protected by being bound to the ribosomes. These short sequences are still fragmentary, and although we do not know the nucleotide sequence(s) responsible for ribosome binding, a pattern of similarities is emerging from among the known binding site sequences, and we may soon be able to explain the specificity of ribosome binding.

Chain Termination

During synthesis the growing polypeptide chain is always bound to the ribosome-mRNA complex by the bond between the last added amino acid and its tRNA molecule, so that there must be some mechanism that permits the release of the polypeptide chain after the end of the message has been reached and the last amino acid added. This mechanism acts by recognising a chain termination (nonsense) triplet at the end of the message (page 213); when this happens the ribosome dissociates into its 50 *S* and 30 *S* components and the peptidyl-tRNA, the mRNA and the completed polypeptide chain are released. The change from 'sense' to 'nonsense' in the middle of a gene also results in chain termination because as soon as the ribosome encounters the nonsense triplet it dissociates and releases the incomplete polypeptide as an N-terminal fragment.

At one time it was thought that the chain-termination triplets (UAA, UAG and UGA) were read by specific tRNA molecules that did not carry amino acids. We now know that this is not so and that two small protein molecules, the so-called release factors, recognise these codons and cause the release of the polypeptide chain. Just how these release factors recognise the chain-termination triplets is still a mystery, but one factor recognises the triplets UAA and UAG, the other UAA and UGA.

Table 14.2 The probable roles of factors in translation

	1. Factors involved in chain initiation
F1	Assists fMet-tRNA to bind to the initiation complex
F2	May assist in the formation of the initiation complex
F3	Required for binding the 30 *S* ribosome subunits to mRNA
	2. Factors involved in chain elongation
TF1	Required for attaching the aminoacyl-tRNA to the ribosome
TF2	Required for translocating the peptidyl-tRNA from the A site to the P site
	3. Factors involved in chain termination
R1	Acts by recognising the chain-termination triplets UAA and UAG
R2	Acts by recognising the chain-termination triplets UAA and UGA

At least seven protein factors have so far been identified as integral elements of the protein synthesising machinery, and they are probably present among the specific ribosomal proteins. The roles of these factors are summarised in table 14.2.

The Intergenic Region

A striking confirmation of the existence of specific initiation and chain termination codons comes from the work of Walter Fiers and his colleagues. In May 1972 they reported the nucleotide sequence of the entire coat-protein gene of phage MS2 and of the intergenic region between this gene and the synthetase gene (page 212). By the end of 1972 they had extended their analysis to include the intergenic region preceding the coat-protein gene and the last forty codons of the 'A protein' gene (figure 14.11). Apart from confirming the sixty-one

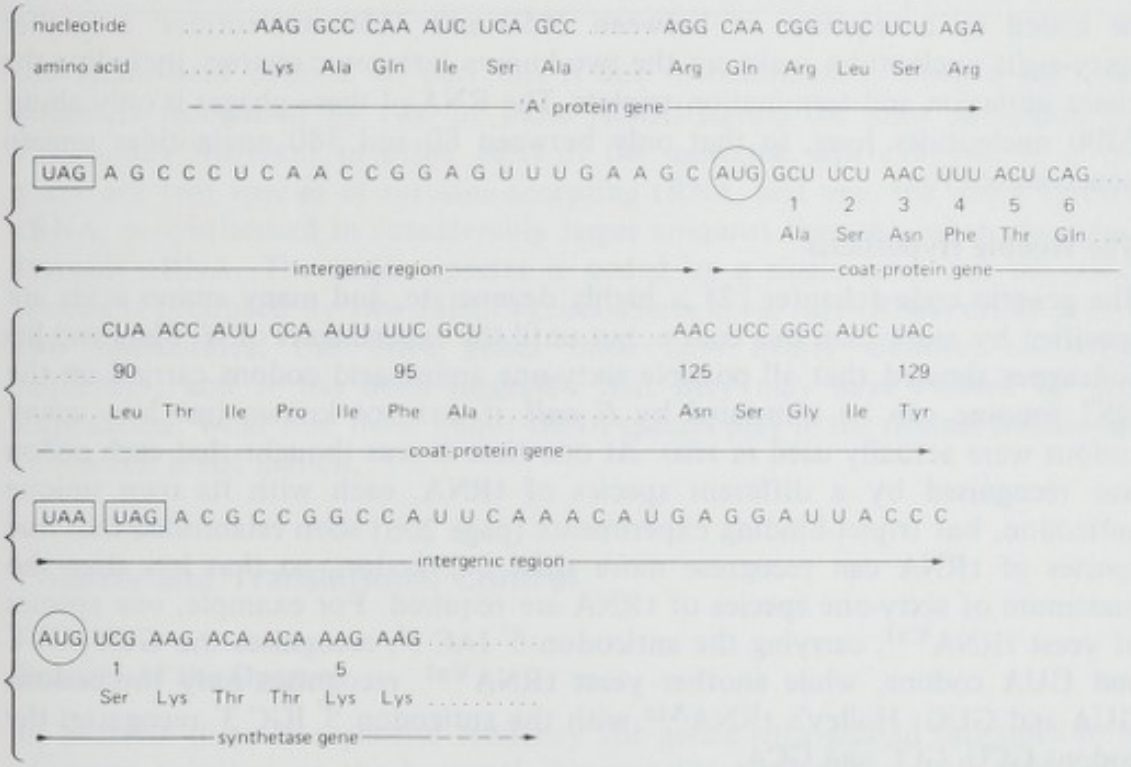


Figure 14.11 Nucleotide and amino-acid sequences in MS2. Recent work has established the exact nucleotide sequence of the last forty codons of the 'A' protein gene, all 129 codons of the coat-protein gene, the first few codons of the synthetase gene, and of the two intergenic regions. Comparison of these sequences with the known amino-acid sequences of the three proteins specified by these genes completely confirms the genetic code as already deduced from *in vitro* studies. Note that the 'A' gene ends with the chain-termination triplet UAG, and the coat-protein gene with two consecutive chain-termination triplets UAA and UAG. Both the coat-protein and synthetase gene sequences are preceded by an AUG initiation codon.

The nucleotide sequences are read continuously from left to right and down the page. Chain-termination triplets are shown boxed and initiation triplets are circled.

codons for the amino acids, these sequence studies reveal that each gene begins with the presumed initiator triplet AUG and ends with one or two chain-termination triplets. Unexpectedly they found that the coat-protein gene ends with the sequence U A A U A G, two consecutive chain-termination triplets; a single chain-termination triplet is usually sufficient to terminate peptide synthesis (as for example in the 'A' gene) so whether or not the double-stop signal represents a built-in safety factor, and how frequently

double-stop signals occur in natural messengers, remains to be seen. Between the chain-termination triplets and initiation triplets is an intergenic region; it is unlikely that this region is translated and it probably contains the sequence of nucleotides responsible for binding the ribosomes.

It is interesting to note that the chromosomes of MS2 and of the closely related phages R17 and Qβ contain only three genes, specifying the 'A' protein required for adsorption on to the host bacterium, the coat protein, and a component of the synthetase required for replicating the viral RNA. These proteins are, respectively, 500-600, 129 and 320 amino acids long, and so must be coded by a sequence of between 2850 and 3150 nucleotides; a further sixty-eight nucleotides make up the two known intergenic regions, including the chain initiation and termination triplets. The RNA of these phages is only about 3300 nucleotides long, so that only between 80 and 380 nucleotides remain unaccounted for.

The Wobble Hypothesis

The genetic code (chapter 12) is highly degenerate, and many amino acids are specified by more than one codon, but until the experiments of W. Fiers and his colleagues showed that all possible sixty-one amino-acid codons carried on the MS2 genome can be translated by *E. coli*, it was not known just how many codons were actually used *in vivo*. At one time it was thought that each codon was recognised by a different species of tRNA, each with its own unique anticodon, but triplet-binding experiments (page 206) soon established that one species of tRNA can recognise more than one codon, so that less than the maximum of sixty-one species of tRNA are required. For example, one species of yeast $tRNA^{Val}$, carrying the anticodon 5′ IAC 3′, recognises the GUU, GUC and GUA codons, while another yeast $tRNA^{Val}$ recognises only the codons GUA and GUG: Holley's $tRNA^{Ala}$ with the anticodon 5′ IGC 3′ recognises the codons GCU, GCC and GCA.

To explain these codon-anticodon relationships, Crick (1966) proposed the so-called wobble hypothesis, based on the observed pattern of degeneracy in the genetic code whereby a change in the first or second base of a codon generally produces a codon representing a different amino acid, whereas a change in the third base frequently produces only another codon for the same amino acid. Thus UCU is one of the codewords for serine—a change in the first U will generate a proline, theonine or alanine codon, a change in the C will produce a phenylalanine, tyrosine or cystine codon but any change of the U in the third position only generates another serine codon; it appears that the first two bases are fixed for a particular amino acid but that the third base is not so rigidly defined. Crick's proposal is that in the third position base of a codon there is a certain amount of non-standard pairing ('wobble') with the corresponding base in the anticodon, so that the anticodon can recognise more than one codon (table 14.3). Thus we can predict that UAC will be the anticodon in the yeast $tRNA^{Val}$ which recognises only the GUA and GUG codons.

We do not know just how many different types of tRNA there are, but in both yeast and *E. coli* there are more than the minimum required to recognise all

Table 14.3 Codon–anticodon relationships according to the wobble hypothesis

Base at 5′ end of anticodon	Corresponding base at 3′ end of codon	
	normal	with wobble
U	A	G
C	G	–
A	U	–
G	C	U
I	C	U A

the amino-acid codons. In part this is due to *redundancy*, when a particular codon is recognised by two or more tRNAs having the same anticodon but structurally different in other parts of the molecule. For example, in *E. coli* there are two species of tyrosine-accepting tRNA, and one, the major tyrosine tRNA, is synthesised in considerably larger amounts than the other, the minor tyrosine tRNA. The major species is coded by a single gene, but the minor species is produced by *two* further genes. Since these Tyr-tRNAs differ in only two nucleotides, the three genes must have nearly identical nucleotide sequences, and it has been suggested that they may have evolved by gene duplication. What role these minor tRNA species play in the normal functioning of the cell is not known.

Polarity and Translational Control

Polarity of the Operon

In bacteria there is a general tendency for genes with related functions to be clustered together on the bacterial chromosome and to be jointly transcribed onto a single species of messenger RNA. Thus, in *E. coli* five genes encoding the proteins involved in the synthesis of tryptophan are transcribed onto a single species of mRNA

$$5'\ \underline{trpE\ trpD\ trpC\ trpD\ trpA}\ 3'$$

while the three genes concerned with the utilisation of lactose are transcribed onto another

$$5'\underline{lacZ\ lacY\ lacA}\ 3'$$

Under normal conditions translation commences only at the 5′ end of the messengers, so that the gene products appear in a temporal sequence, the *trpE* protein before the *trpD* protein, the *lacZ* protein before the *lacA* protein and so on. If each gene is to produce a separate polypeptide and the intergenic region is not to be translated the polygenic messengers must be 'punctuated', and the end

of each gene must be marked by a chain-termination triplet, followed by the initiation sequence of the succeeding gene (see figure 14.11). We still do not know how reinitiation takes place, but one *possible* mechanism is that the ribosome commences translation, say of the *trp* messenger, and on reaching the terminator at the end of the *trpE* gene the 50 *S* ribosomal subunit detaches, releasing the *trpE* polypeptide; the 30 *S* subunit now continues along the messenger until it reaches the next initiation sequence, when another 50 *S* subunit attaches and translation of the *trpD* gene can commence. The products of the five *trp* genes are produced in equimolar amounts so that once translation commences it probably proceeds to the 3′ end of the messenger. However, this is not always so, as the products of the *lacZ*, *lacY* and *lacA* genes are produced in the ratio 5:2:1. The mechanism that gives rise to this natural polarity is not understood but it is worth noting that *lacZ*, at the 5′ end of the mRNA, is translated the most often and *lacA*, at the 3′ end, the least often; this could mean that when a ribosome reaches the *lacZ* (or *lacY*) termination triplet it sometimes irreversibly dissociates from the mRNA before it can reinitiate translation at the succeeding initiation signal.

Polar Mutations

An interesting consequence of this polarity is the occurrence of *polar mutations*, first discovered in the lactose gene cluster. A polar mutation terminates protein synthesis in the gene where it occurs *and* reduces the quantities of the proteins encoded by the more distal genes. A strain with a polar mutation in *lacZ* produces no *lacZ* protein and only reduced amounts of the *lacY* and *lacA* proteins; if the mutation is in *lacY*, then there is a normal amount of active *lacZ* protein, no active *lacY* protein and a reduced amount of *lacA* protein. These polar mutants are due to the generation of a nonsense (chain termination) codon, either by a direct change from sense to nonsense or by a frameshift mutation producing a more distal nonsense triplet.

Polar mutations are either *weak*, when the quantity of distal protein (for example *lacA* product) is not severely reduced, or *strong*, when very little distal protein is produced (figure 14.12). The severity of the polar effect seems to depend on the position of the mutation within the proximal gene. A polar mutation at the proximal (5′) end of the *trpE* gene is strongly polar and only produces about 5 per cent of the other *trp* encoded proteins, whereas a similar mutation near the other end of *trpE* is only weakly polar and produces about 60 per cent of the other *trp* proteins. The same applies to polar mutations in *lacZ*. What seems to be important is how near the polar mutation is to the next initiation sequence, as a strain with a strong polar mutation in *lacZ* can be made weakly polar by deleting a large segment of *lacZ* between the mutant site and the distal end of the gene—in effect, bringing the polar mutation nearer to the initiation sequence of the *lacY* gene; similar deletions at the proximal end of *lacZ* have no effect on the degree of polarity.

The most favoured explanation is that when the ribosome reads a nonsense codon the 50 *S* subunit detaches, and the 30 *S* subunit 'wanders' to and fro

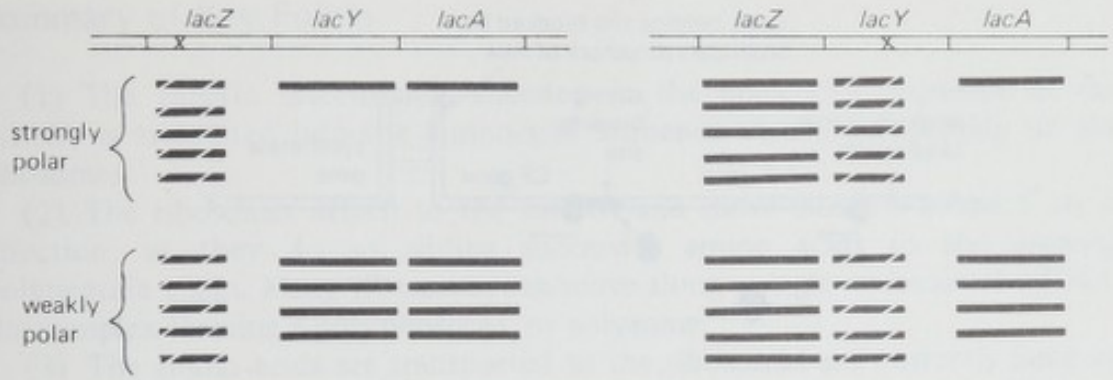


Figure 14.12 Polar mutations in the lactose operon of *E. coli*. Polar mutants of the *lacZ* gene produce an incomplete and inactive *lacZ* protein (short hatched lines) but some active *lacY* and *lacA* proteins (longer unbroken lines). *lacY* polar mutants (right) produce normal amounts of *lacZ* protein, no active *lacY* protein and a reduced amount of *lacA* protein.

The strongly polar mutants (top) produce less distal protein than do the weakly polar mutants (below).

along the messenger until either a new initiation sequence is encountered or the 30 *S* subunit dissociates from the messenger template; the nearer the nonsense mutation to the next initiation sequence, the greater will be the chance of reinitiation occurring before dissociation and the weaker will be the polar effect.

Translational Control in the Small RNA Phages

The RNA of the small phages R17, Qβ and MS2 serves both as a chromosome and as a template for translation, and when an *E. coli* host cell is infected the three phage genes are differentially translated; not only are many more coat-protein molecules made than of either the A-protein or RNA synthetase component, but the synthetase gene ceases to function about ten minutes after infection. This is because of control at the level of translation.

Many nonsense mutations in the coat-protein gene are polar, and abolish or reduce the activity of the synthetase gene (depending on whether the mutations are proximal or distal) but have no effect on the synthesis of the A-protein. On the other hand, nonsense mutations in the synthetase or A-protein genes do not show any polar effect. The coat-protein and A-protein genes are translated independently because, following infection, the ribosomes attach to two binding sites, one at the start of the A gene and one at the start of the coat-protein gene; these genes are translated at different rates because the coat-protein binding site has a greater ribosomal affinity than the A binding site–as a result, the coat-protein gene is translated the more often.

The phage mRNA has an ordered secondary structure and the polar effect, whereby the synthetase gene is only translated after translation of the coat-protein gene, is thought to be because the initiation sequence of the synthetase gene lies within a hairpin loop and is not available as a ribosomal binding site until the ribosome has moved along the preceding coat-protein gene and 'opened up' the loop to form single-stranded mRNA (figure 14.13). This also accounts for the proximal coat-protein nonsense mutations being more

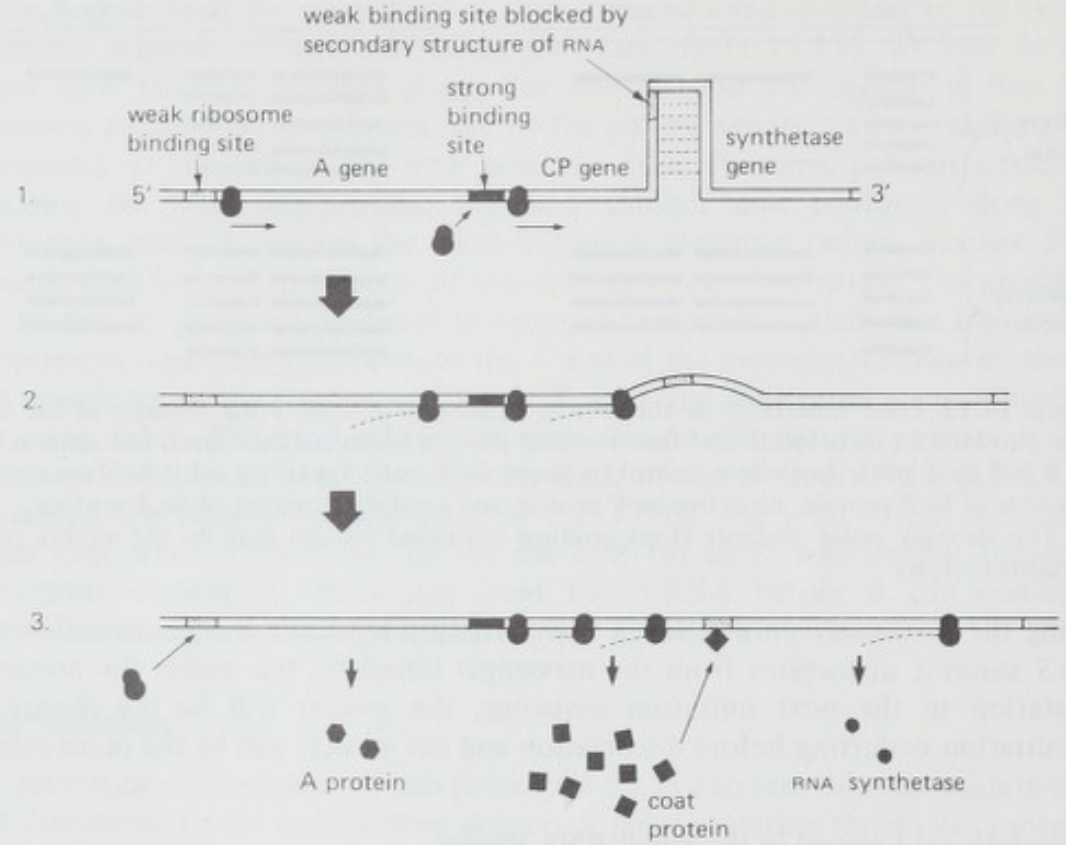


Figure 14.13 Translational control in phage R17.

(1) Ribosomes attach to 'A' and coat protein gene-binding sites and commence to translate these genes.

(2) Movement of the ribosome along the coat-protein gene opens up the secondary structure of the RNA, making available the synthetase gene-binding site. Translation of the A and coat-protein genes continues.

(3) When sufficient synthetase has been made, a molecule of coat protein attaches to the synthetase gene and represses further gene activity.

strongly polar than distal nonsense mutations as a ribosome that 'stops' at the proximal end of the coat-protein gene will have less chance of opening up the hairpin loop at the beginning of the synthetase gene than a ribosome that proceeds nearly to the end of the coat-protein gene. Even when a ribosome has translated the coat-protein gene, there is only about a one in ten chance that it will continue and translate the synthetase gene, probably because the synthetase initiation sequence has a comparatively low ability for binding ribosomes. Finally, the synthetase gene is turned off about ten minutes after infection, when enough RNA synthetase has been made to enable the replication of the phage RNA, because the phage-coat protein can bind to the synthetase gene and repress the synthesis of further synthetase molecules; a clear indication of the existence of this control mechanism is that phage mutants which do not produce an active coat protein, because of a non-polar mutation within the coat-protein gene, produce greatly increased amounts of the synthetase because the gene cannot be switched off.

Summary of Key Points

(1) The genetic information encoded in the nucleotide sequence of the mRNA is translated into the amino-acid sequence of the polypeptide on the ribosomes.

(2) The ribosomes attach to the mRNA and move along it in the 5′ to 3′ direction, as they do so adding successive amino acids to the growing polypeptide chain. Many ribosomes can move along a single molecule of mRNA, the complex forming a polyribosome, or polysome.

(3) The amino acids are transported to the ribosomes and correctly lined up on the mRNA template by highly specific adaptor molecules of transfer RNA.

(4) For each amino acid there is at least one highly specific type of tRNA which recognises the amino acid on the one hand and a particular mRNA codon on the other.

(5) Each tRNA becomes charged with its own amino acid by a specific aminoacyl synthetase and moves to the ribosomes; each molecule of aminoacyl-tRNA now recognises the appropriate mRNA codon because it contains an anticodon nucleotide sequence, which can form complementary base pairs with the mRNA codon.

(6) Specific nucleotide sequences signal the start and finish of the genetic message to be translated.

(7) Specific protein factors are involved in the initiation, elongation and termination of polypeptide synthesis.

(8) Polar mutations abolish the activity of the gene in which they occur and reduce the activity of more distal genes in the same operon. The nearer a polar mutation to the next initiation triplet the less is the polar effect.

(9) Differential gene translation can also be achieved by (i) different binding sites having different affinities for the ribosomes, and (ii) a ribosome binding site being blocked by the secondary structure of the RNA being transcribed until a more proximal gene has been transcribed.

15 The Regulation of Gene Activity in Procaryotes

Good order is the foundation of all things.
Edmund Burke (1729–1797)

Although there is very good reason to believe that in most higher organisms every cell contains a complete set of genetic information it would be extremely wasteful if every gene was active all the time; clearly the structural protein keratin, found in nails, horns and hoofs, is not required by the secretory cells of the pancreas, whose main function is to produce certain of the enzymes required for the digestion of food. Not only do different types of cell carry out different sets of biochemical reactions, but the same cell may carry out different reactions according to the available substrates or the particular stage in development of an organism. Clearly, there must be some regulatory mechanism, that enables genes to be turned on and off so that the proteins they specify are only synthesised when actually required. This temporal control is very clearly seen in the salivary gland cells of the larvae of *Drosophila melanogaster.* These cells contain giant polytene chromosomes and particular genes or groups of genes can be identified by the particular pattern of lateral banding. Sometimes a particular band seems to swell up and become diffuse, forming a *puff.* These puffs, and only the puffs, are actively synthesising RNA; this RNA is almost certainly messenger, so that the presence of a puff indicates the activity of the particular gene or group of genes located in the band. During larval development and pupation there is a very precise and predictable sequence of puffing, indicating that as development proceeds some genes are being switched off and others are being switched on.

We still know comparatively little about the molecular basis of genetic regulation in eucaryotic cells (this is discussed in chapter 18), and the most detailed knowledge on genetic regulation has come from studies with micro-organisms, largely because of the comparative ease with which particular proteins can be assayed and isolated and genetic analysis carried out. In procaryotes, just as in eucaryotes, the genes must be regulated if cellular components are only to be synthesised when actually required, but whether or not similar systems to those found in procaryotes will eventually be found in eucaryotes still remains to be seen.

Feedback Inhibition

In many biochemical pathways leading to the production of *small* essential metabolites, such as the amino acids, a fine degree of regulation is achieved by

feedback inhibition. This control, which enables the rate of a reaction to be turned up or down, is at the biochemical rather than the genetic level and some of the end product of the pathway is fed back into the system so as to inhibit the activity of the *first* enzyme in its own pathway. In *Salmonella,* histidine is synthesised from phosphoribosyl pyrophosphate and ATP in ten consecutive steps (figure 15.1), and when an adequate amount of histidine is present in the cell some of it is diverted from being incorporated into protein and is fed back

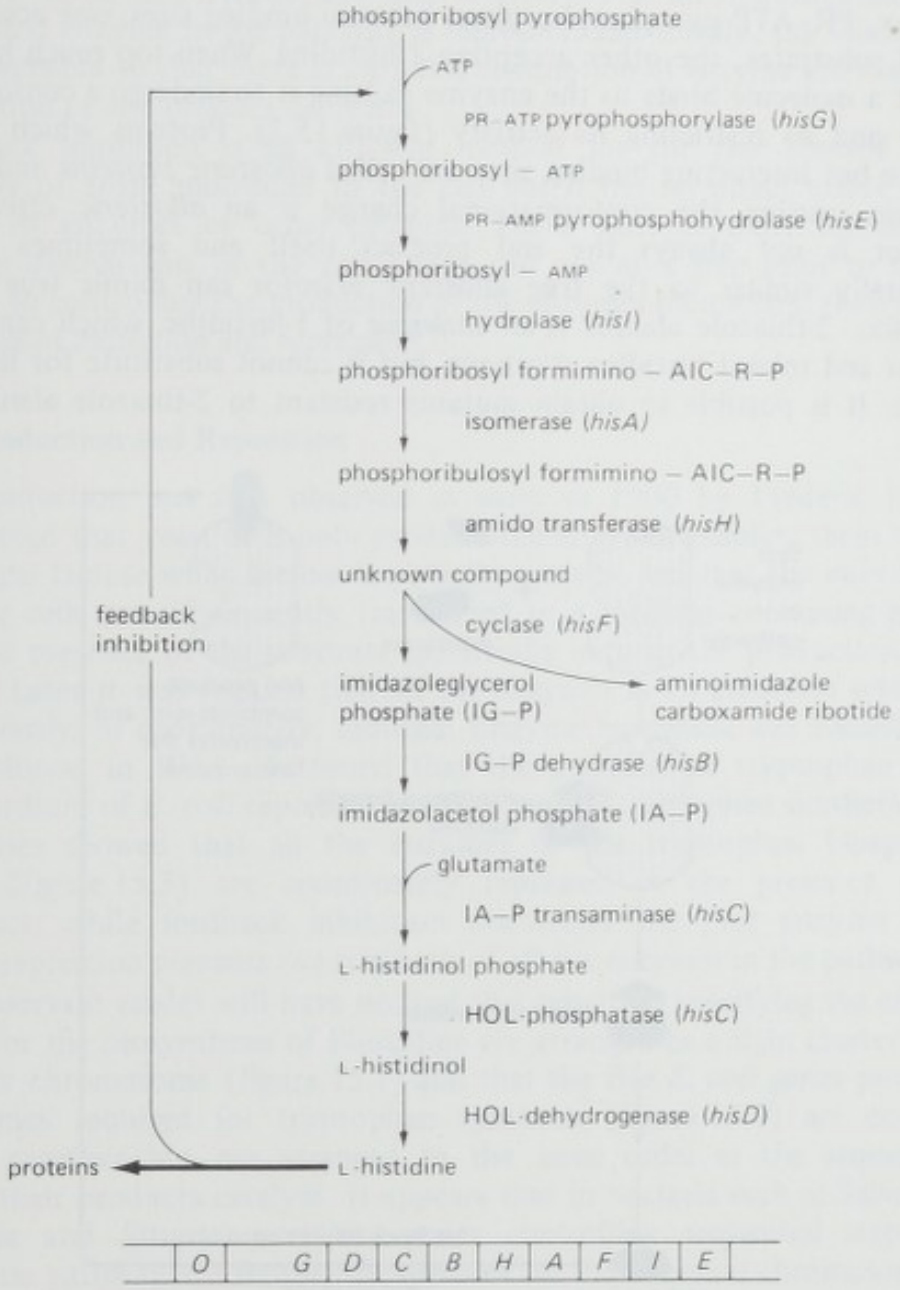


Figure 15.1 Histidine biosynthesis in *Salmonella typhimurium.* Showing the sequential steps in the biosynthesis of histidine, the enzymes catalysing these reactions, the histidine genes specifying these enzymes and (below) the linkage map of the histidine operon. The end product, l-histidine, is (1) incorporated into proteins and (2) fed back to inhibit the further biosynthesis of histidine.

to inhibit the activity of PR-ATP pyrophosphorylase. Feedback inhibition (sometimes called end-product inhibition) is analogous to negative feedback in an electronic circuit, where a small part of the output of a valve is fed back into a previous valve in the same circuit. If the output of the last valve increases, the feedback signal also increases and causes a *decrease* in the output of the first valve, and vice versa; this ensures that the output signal of the circuit remains constant, irrespective of the input signal.

The explanation of feedback inhibition is that the first enzyme in the pathway, PR-ATP pyrophosphorylase, has two binding sites, one accepting the normal substrates, the other accepting L-histidine. When too much histidine is present a molecule binds to the enzyme causing it to undergo a conformational change and so restricting its activity (figure 15.2). Proteins which have two separate but interacting binding sites are called *allosteric proteins* and the small molecule causing the conformational change is an *allosteric effector*. The inhibitor is not always the end product itself and sometimes molecules structurally similar to the true allosteric effector can mimic true feedback inhibition; 2-thiazole alanine is an analogue of L-histidine, which can act as an effector and inhibit histidine synthesis, but it cannot substitute for histidine in protein. It is possible to obtain mutants resistant to 2-thiazole alanine and it

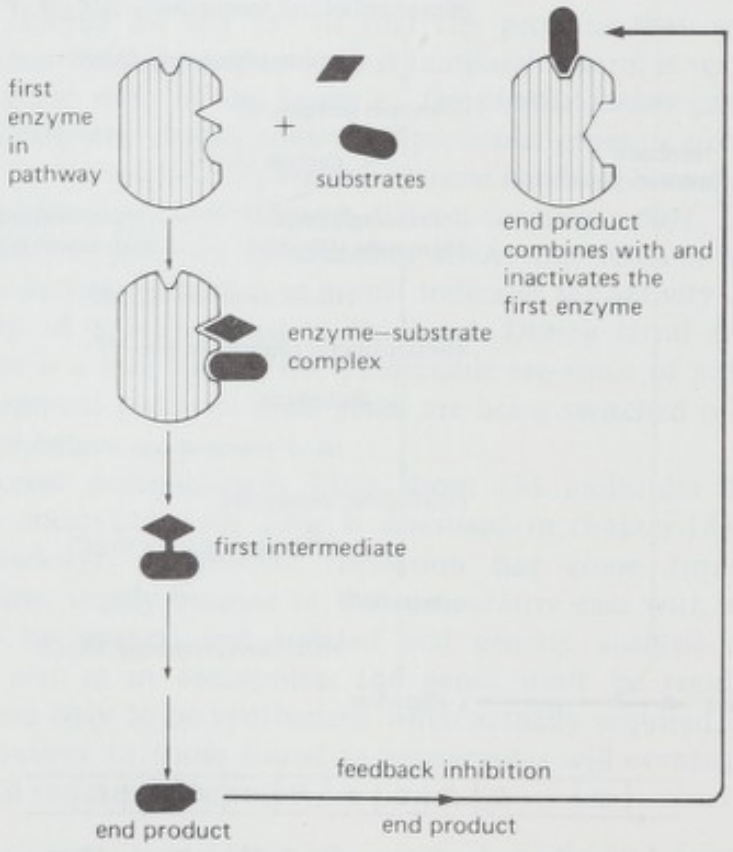


Figure 15.2 Feedback inhibition. The enzyme is an allosteric protein with two separate and interacting binding sites. The end product of the pathway is an allosteric effector, which binds to one of these sites and causes a conformational change in the enzyme so that it no longer has catalytic activity.

turns out that these mutants are due to an alteration in the gene specifying PR-ATP pyrophosphorylase, modifying the enzyme so that while its catalytic activity is retained it can no longer bind the allosteric effector. This could have serious consequences for the cell as the unnecessary synthesis of phosphoribosyl-ATP could seriously deplete the amount of ATP available for use in other biochemical reactions in the cell.

There are three features of control by feedback inhibition: firstly, only the end-product itself or a related analogue is effective as an inhibitor; secondly, only the first enzyme in the pathway is inhibited; and thirdly, the inhibition is usually reversible so that there is a gradual resumption of enzyme activity as the concentration of end-product decreases.

Feedback inhibition is an efficient method for the *fine control* of the production of *small* molecules in the cell but it has no direct effect on the synthesis of enzymes or other proteins. There must be more sophisticated regulatory mechanisms in the cell which can act at a step prior to enzyme synthesis, so controlling the production of the enzymes themselves; these are the phenomena of *induction* and *repression.*

Enzyme Induction and Repression

Enzyme induction was first observed as early as 1900 by Frédéric Dienert, who observed that yeast cells only produce the enzymes enabling them to grow on the sugar lactose when lactose is actually present, and that the enzymes are lost if the cells are subsequently transferred to a medium containing glucose; hence, the presence of the substrate specifically *induces* the production of the enzymes. Later it was realised that all the enzymes in an inducible system are simultaneously, or *coordinately,* induced. Enzyme repression was discovered by Jacques Monod in 1953. He found that the presence of tryptophan in the growth medium of *E. coli* repressed the synthesis of tryptophan synthetase, and later studies showed that all the enzymes in the tryptophan biosynthetic pathway (figure 15.3) are coordinately repressed in the presence of the end-product; while feedback inhibition *inactivates* the *first* enzyme in the pathway, repression *prevents the synthesis* of *all* the enzymes in the pathway.

The observant reader will have noticed that ten genes specifying the enzymes required for the biosynthesis of l-histidine are arranged in a tight cluster on the *Salmonella* chromosome (figure 15.1) and that the five *E. coli* genes producing the enzymes required for tryptophan synthesis (figure 15.3) are not only clustered together but are arranged in the same order as the sequence of reactions their products catalyse. It appears that in bacteria such as *Salmonella, Escherichia* and *Streptomyces* the genes controlling sequential steps in a biosynthetic pathway are frequently adjacent on the bacterial chromosome and can be coordinately repressed; this is in contrast to eucaryotes where related genes appear to be scattered throughout the genome, and although this does not preclude coordinate control it makes it more difficult to achieve. This clustering of bacterial genes was first observed in 1956 by Miloslav Demerec and his colleagues but it remained unexplained until 1961, when Jacques Monod and

François Jacob, working at the Institut Pasteur, Paris, produced their elegant and far reaching *operon* model for the genetic control of protein synthesis. This stimulating hypothesis accounted for many previously unexplained facts of the regulation of protein synthesis; furthermore, it was capable of experimental proof and opened up a large new area of experimental research. Most of the major concepts of operon structure and function have come from this and subsequent work on the lactose operon of *E. coli*, and for this far-sighted work Monod and Jacob, together with their director André Lwoff, were awarded the Nobel prize for medicine in 1965.

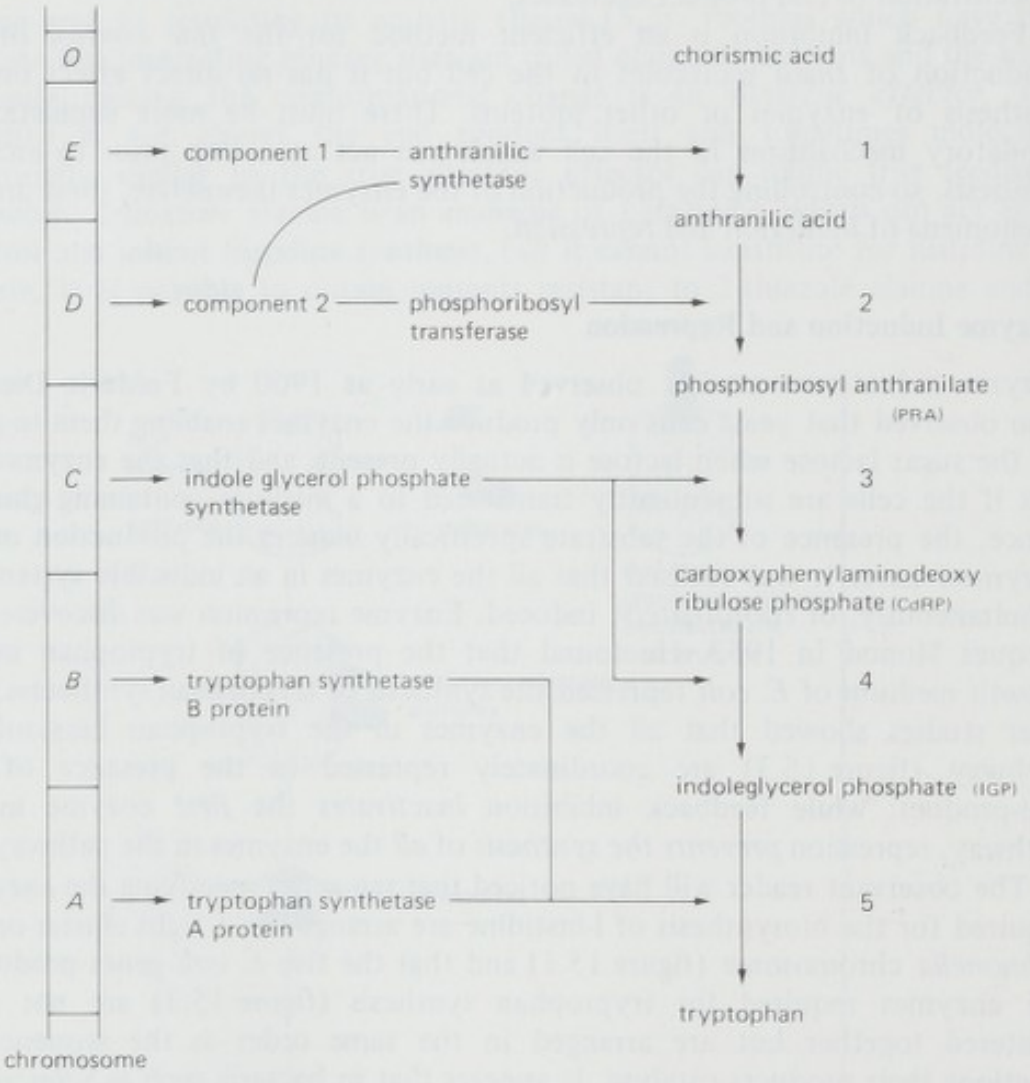


Figure 15.3 The biosynthesis of tryptophan in *Escherichia coli*. The synthesis of tryptophan from chorismic acid is a sequence of five reactions. The protein specified by the *trpE* gene (ASase component 1) has no activity unless it is bound to the *trpD* gene product (ASase component 2) to form anthranilic synthetase. Reaction 1 is catalysed by the complex enzyme anthranilic synthetase, but reaction 2 is catalysed by ASase component 2 (otherwise known as PR transferase) alone. Reactions 3 and 4 are catalysed by IGP synthetase, the product of the *trpC* gene, while reaction 5 requires tryptophan synthetase, a complex of the products of the *trpB* and *trpA* genes.

The Operon Model

Jacob and Monod's hypothesis was based on two premises: first, that there are two fundamental types of genetic unit, the so-called *structural genes*, concerned with elaborating the structures of enzymes and other cellular proteins, ribosomal RNA and transfer RNA, and *regulatory genes* which act so as to control the activity of the structural genes; second, that the genetic material is organised into a number of separate units of coordinate expression, the *operons* –thus the five closely linked structural genes specifying the enzymes involved in tryptophan biosynthesis in *E. coli* (figure 15.3) would, together with their operator and promoter genes, constitute the tryptophan operon.

These studies, mainly carried out on the lactose operon of *E. coli*, revealed that there were four principal components involved in the regulation of gene function: (i) a group of structural genes (SGs) whose activity is regulated; (ii) a *regulator* gene (RG) specifying a repressor substance and which may or may not be linked to the group of structural genes; (iii) a repressor substance; and (iv) an *operator* region, *O*, at one end of the group of structural genes, sensitive to the repressor and acting as a switch to turn on and off the activity of the structural genes.

The operon model easily accounts for both enzyme induction and enzyme repression. In an inducible system, such as the lactose operon, the product of the regulator gene, termed the *repressor*, can recognise and attach to the operator region and can block transcription either by hindering the movement of RNA polymerase along the DNA template or by preventing RNA polymerase from binding to the DNA. (The terms aporepressor and apoinducer (in positive control systems) are often used to describe gene-specified repressors or inducers, while corepressor and coinducer (in repressible systems) are used to describe repressing or inducing metabolites.) This repression occurs in the absence of substrate, but when substrate is present it acts as an *inducer* by combining with and inactivating the repressor; this repressor complex can no longer recognise and attach to the operator so that the structural genes are switched on and transcription commences (figure 15.4). In a repressible system, such as the tryptophan operon, the repressor is only active when it is combined with a repressing metabolite, or corepressor; when the end product (corepressor) is present, it combines with the repressor to produce an active repressor complex which attaches to the operator and switches off the structural genes. The essence of the model is that the repressor can exist in either an active or an inactive state; in an inducible system it is *inactivated* by combining with a specific *inducer*, while in a repressible system an *inactive* repressor is *activated* by combining with a corepressor.

The operon model thus permits regulation at the level of the immediate gene products, so that mRNA and protein are only produced when they are actually required by the cell. It is an essential part of the model that each repressor must only be able to combine with specific substrate or end product molecules and that each active repressor must be able to recognise the specific nucleotide sequence of its own operator region.

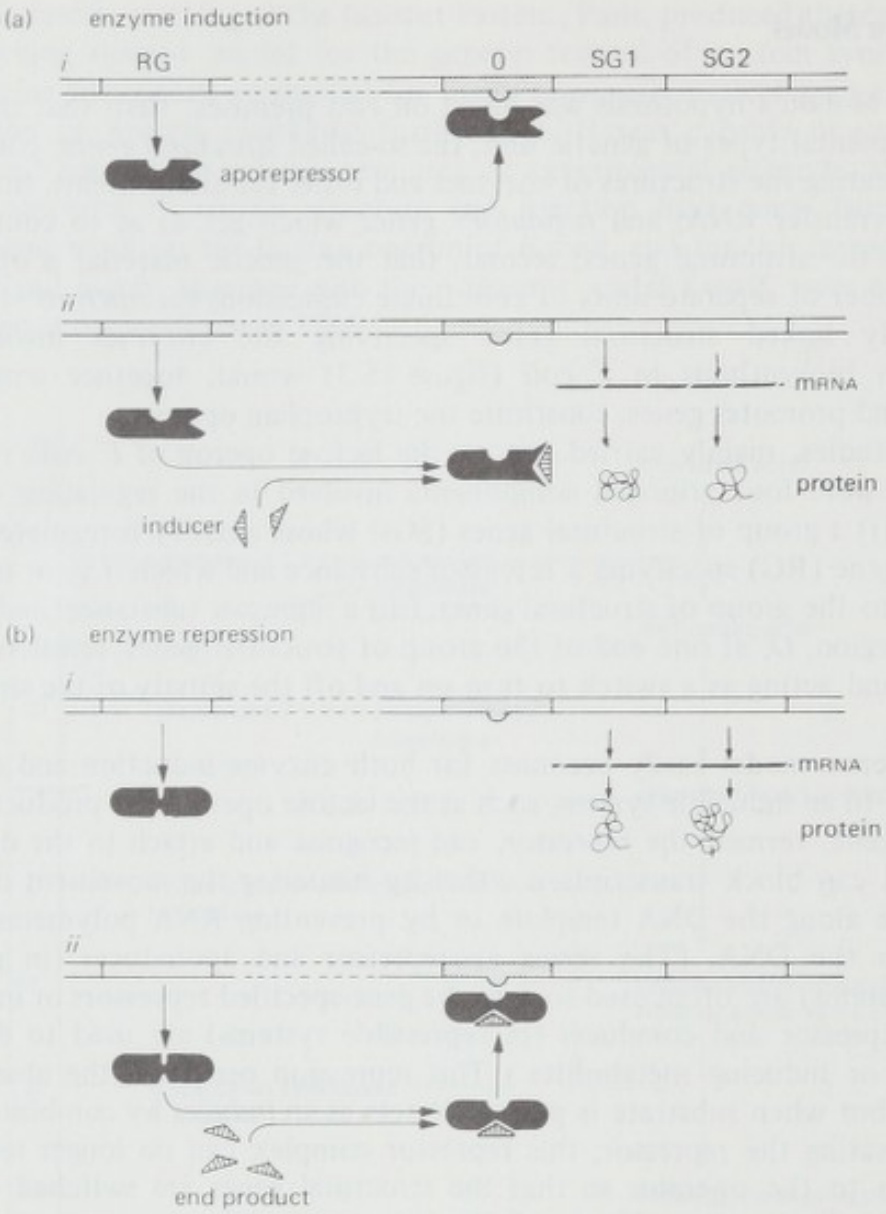


Figure 15.4 The operator–repressor model for enzyme induction and repression.

(a) Enzyme induction

i. Aporepressor binds to the operator and switches off the structural genes.

ii. When substrate (inducer) is present it binds to and inactivates the aporepressor. The repressor complex cannot bind to the operator and the structural genes are switched on.

(b) Enzyme repression

i. The aporepressor is unable to bind to the operator, and so the structural genes are switched 'on'.

ii. When end product is present it binds to the repressor. The repressor complex is now able to recognise and attach to the operator, switching 'off' the structural genes.

Note that in their 1961 paper Jacob and Monod did not recognise the necessity for a promoter region (pages 225 and 265), envisaging that the operator was the initiation point for the transcription of the several linked structural genes, and they incorrectly speculated that the repressor, now known to be protein, was RNA.

The Lactose Operon of 'E. coli'

Structure and Function

E. coli is able to use the disaccharide sugar lactose (glucose-4-β-D-galactoside) as its sole carbon source provided that lactose can enter the cell and that it can be broken down into its two component monosaccharides, galactose and glucose. The proteins that carry out these functions are specified by the structural genes of the lactose operon. This operon is located between the *proB* (ability to synthesise proline) and the *tsx* (resistance to phage T6) loci and consists of three structural genes, *lacZ*, *lacY* and *lacA*, with their own promoter (*lacP*), operator (*lacO*) and terminator (*t*) (figure 15.5). (Many *lac* operon geneticists, it should be noted, do not conform to conventional notation and refer to the *lac* genes by lower case rather than by capital letters.)

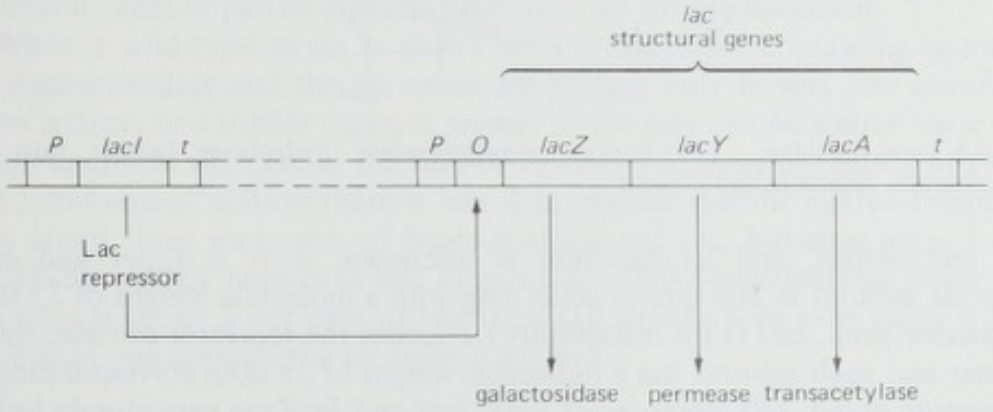


Figure 15.5 The lactose operon of *E. coli* and its regulator gene. The three *lac* structural genes are transcribed as a single unit by the RNA polymerase binding to the promoter (*P*) and transcribing as far as the terminator (*t*) at the end of the structural genes. These genes are not transcribed if *lac* repressor protein is bound to the operator gene (*O*), but if lactose is present it acts as a co-inducer by binding to the repressor in such a way that the repressor can no longer attach to the operator and the structural genes are transcribed.

The repressor is produced by the closely linked *lacI* gene; this is a separate unit of transcription and has its own promoter and terminator.

The *lacY* gene specifies galactoside permease, a membrane-bound protein (it has not been shown to be an enzyme) which permits the transfer of lactose across the osmotic barrier of the cell membrane. This protein specifically binds galactosides and has a molecular weight of about 30 000, corresponding to a polypeptide of about 260 amino acids.

The *lacZ* gene controls the production of β galactosidase, the enzyme which hydrolyses lactose into galactose and glucose (figure 15.6). This enzyme is a tetramer, and each of the four subunits is a polypeptide about 1172 amino acids long with a molecular weight of about 135 000.

In addition to *lacZ* and *lacY*, which encode proteins essential for lactose utilisation, the lactose operon contains the *lacA* gene, specifying a third protein, thiogalactoside transacetylase. This protein does not appear to be essential for

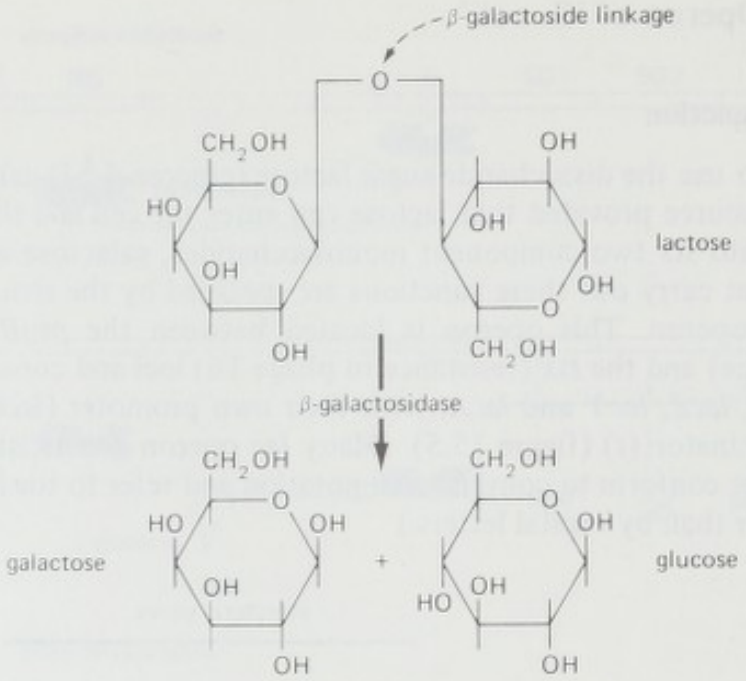


Figure 15.6 β-galactosidase. The enzyme β-galactosidase hydrolyses lactose into the monosaccharides galactose and glucose.

lactose metabolism, and its function is unknown; it is a dimer and each polypeptide subunit is 268 amino acids long with a molecular weight of 32 000. The regulator gene, *lacI* (I for inducibility), encodes the repressor protein; this is a tetramer and each subunit has a molecular weight of 38 000, corresponding to a polypeptide about 330 amino acids long. *lacI* and *lacZ* are very closely linked and are separated by not more than 100 nucleotide pairs.

The three structural genes *lacZ*, *lacY* and *lacA* code for polypeptides containing a total of about 1700 amino acids, so that the minimum length of these genes is about 5100 nucleotide pairs. The regulator gene, *lacI*, accounts for a further 1000 nucleotide pairs so that the operon has an overall minimum length of 6100 nucleotide pairs–about 0.2 per cent of the *E. coli* genome.

Mutations within the regulatory elements of the *lac* system (that is, the operator and promoter regions and the *lacI* gene) were first recognised because they *coordinately* affected the functioning of *lacZ*, *lacY* and *lacA* whereas mutations within the structural genes did not have this effect. For example, a missense mutation in a structural gene causes a single amino-acid substitution in the peptide specified by the mutant gene, but as neither transcription nor translation is interfered with, the functioning of the other genes in the operon is not affected. A *lacZ* missense mutant produces an altered *lacZ* polypeptide, and although there may be little or no β-galactosidase activity the *lacY* and *lacA* genes will continue to produce fully active permease and *trans*-acetylase. A nonsense mutation in a structural gene causes the premature termination of translation so that the mutant gene does not produce any active protein, and if the mutation is at the operator end of a cluster of coordinately controlled genes there may be a polar effect (page 248). A *lacZ* polar nonsense mutation produces

no active β-galactosidase, but it does produce *reduced* amounts of permease and *trans*-acetylase. A frameshift mutation nearly always generates a nonsense triplet and so behaves as a nonsense mutant. Thus, with the exception of certain polar mutants of *lacZ* where the polar effect is so extreme that there is no detectable *lacY* or *lacA* activity, all mutations within the structural genes have different effects on the gene in which they occur and on the other structural genes within the operon. By way of contrast, the operator–repressor mechanism acts as a switch to turn on and off the activity of the structural genes under its control, so that a mutation in one of its elements has a coordinate effect on all these structural genes.

In general, mutations within the regulatory elements are distinguished from mutations within the structural genes by three criteria: (i) their location within the genetic segment comprising the *lac* operon, (ii) the effects of the mutations on the expression of each of the structural genes, and (iii) the expression of the structural genes in partial diploids heterozygous for the mutation.

When a wild-type strain is grown on a medium not containing lactose both the β-galactosidase and the permease are present only in very low amounts, but when lactose, or a similar sugar, is present as the sole carbon source there may be a thousand-fold increase in the rate of synthesis of these proteins. For example, in a non-induced culture there is less than one *lac* specific mRNA template and only about three molecules of β-galactosidase per cell, but after induction there are 35–50 mRNA templates and as many as 3000 β-galactosidase molecules per cell (this is about 3 per cent of the total cell protein)—or even more if there is more than one copy of the *lac* operon present.

Mutations in the *lacI* Regulator Gene

The repressor protein specified by $lacI^+$ can, on the one hand, interact with a specific operator site adjacent to *lacZ* and so prevent transcription of the *lacZ*, *lacY* and *lacA* structural genes, or, on the other hand, it can recognise and bind the lactose inducer, changing the conformation of the repressor complex so that it can no longer bind to the operator; mutations within *lacI* can influence either of these interactions.

The first *lacI* mutants were recognised because they had lost the ability to be induced and so produced the *lacZ*, *lacY* and *lacA* proteins even in the absence of an inducer. These *constitutive* I^- mutants (note that the *lac* prefix is frequently omitted when designating the lactose operon genes) no longer produce an active repressor, because of either a deletion or a nonsense mutation within *lacI*, or because a missense mutation has caused a conformational change in the repressor so that it can no longer recognise and bind to the nucleotide sequence of the operator (figure 15.7a). The activity of these *I* alleles was investigated by making partial diploids with one set of *lac* genes on the bacterial chromosome and a second set on an F-prime factor (see page 132). An I^-Z^+ mutant produces β-galactosidase constitutively as no repressor can be made and so the Z^+ gene cannot be turned off, but when an F factor carrying I^+Z^- (that is, F' $lacI^+Z^-$) is introduced into it, the production of β-galactosidase falls to zero and thereafter

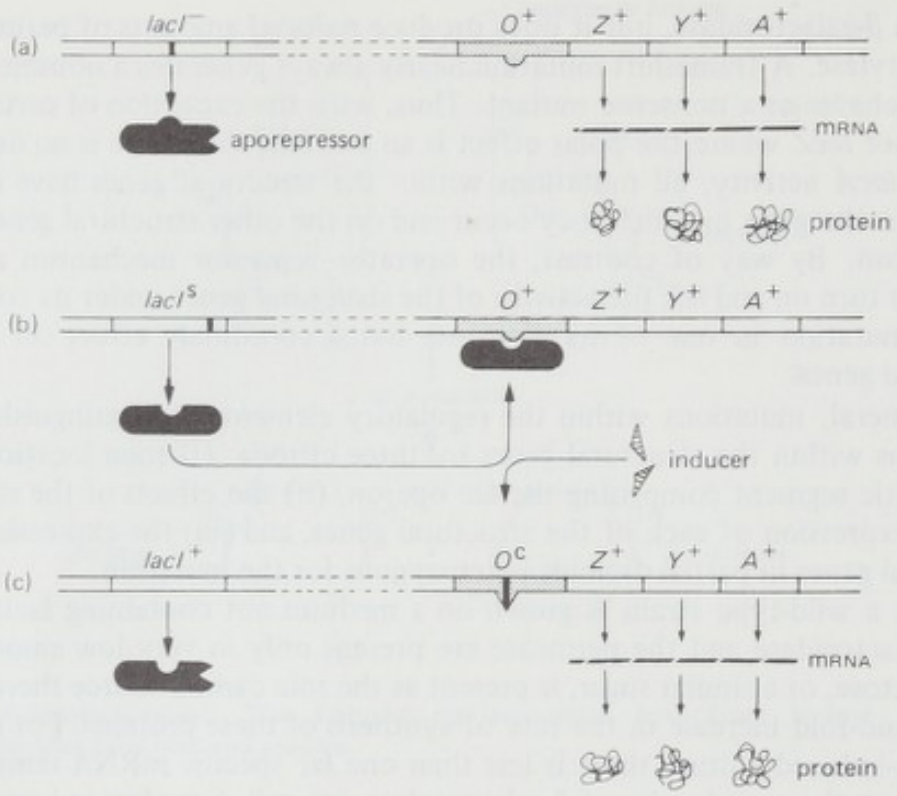


Figure 15.7 Regulatory mutations in the *lac* system of *E. coli*. The effect of the $lacI^-$, $lacI^S$ and $lacO^C$ mutations on the transcription of the *lac* structural genes.

(a) $lacI^-$ specifies an altered aporepressor, unable to bind to the operator because of a conformational change; the structural genes are permanently switched on.

(b) In $lacI^S$ strains the aporepressor cannot be inactivated by inducer so the structural genes are permanently switched off.

(c) In $lacO^C$ mutants the operator is altered so that it cannot bind the aporepressor; the structural genes are permanently switched on.

the partial diploid $I^-Z^+/F\ lacI^+Z^-$ behaves as a normally inducible strain (table 15.1). Likewise, when F $lacI^-Z^-$ is introduced into a wild-type I^+Z^+ strain (I^+Z^+/F $lacI^-Z^+$) β-galactosidase production remains inducible. These experiments tell us two things. Firstly, I^+ is dominant over I^- in either the *cis* (I^+Z^+/I^-Z^-) or the *trans* (I^+Z^-/I^-Z^-) arrangements; if I^- were dominant β-galactosidase synthesis in the partial diploids I^+Z^+/I^-Z^- and I^+Z^-/I^-Z^+ would have remained constitutive and inducible respectively. Secondly, in the *trans* arrangement the I^+ gene and the Z^+ gene, whose activity is controlled, are on different chromosomes so that I^+ must specify a diffusible cytoplasmic repressor substance.

Another mutation within *lacI*, known as I^S, results in the production of a repressor with a greatly reduced affinity for binding the inducer molecule so that even when inducer is present the repressor cannot be inactivated and the operator remains permanently switched off (figure 15.7b). This mutation is dominant to I^+ and I^SZ^+/F $lacI^+Z^+$ partial diploids do not produce β-galactosidase and so are unable to ferment lactose; although I^+ produces normal repressor the I^S allele produces an altered repressor (*super-repressor*) which cannot be inactivated.

Table 15.1 Mutations in the *lac* operon and the *lacI* gene of *E. coli* and their effect on gene expression

(a) Mutations in the *lac* structural genes

Mutation	Effect of mutation	Activity of *lac*			Comment
		Z	*Y*	*A*	
missense in *Z*	inactivates *lacZ*	O	I++	I++	no coordinate effect
missense in *Y*	inactivates *lacY*	I++	O	I++	
missense in *A*	inactivates *lacA*	I++	I++	O	no effect on inducibility
nonsense in *Z*	extreme polar mutation	O	O	O	The more proximal the mutation, the stronger is the polar effect
nonsense in *Z*	polar mutation	O	I+	I+	
nonsense in *Y*	polar mutation	I+	O	I+	All can be suppressed by nonsense suppressors

(b) Mutations in the *lac* regulator genes

Mutation	Effect of mutation	Activity of *lac*			Phenotype of partial diploids		Comment
		Z	*Y*	*A*			
lacI$^-$	repressor cannot bind to operator	C	C	C	I^+Z^+/I^-Z^-	normally inducible	I^+ is dominant over I^- in both *cis* and *trans*
					I^+Z^-/I^-Z^+	normally inducible	
lacI^S	repressor cannot bind to inducer and so is permanently bound to operator	O	O	O	I^SZ^+/I^+Z^-	no activity	I^S is dominant over I^+
					I^SZ^-/I^+Z^+	no activity	
lacO^C	operator cannot bind aporepressor and is permanently switched on	C	C	C	O^CZ^+/O^+Z^-	constitutive	O^C is *cis* dominant but *trans* recessive
					O^CZ^-/O^+Z^+	normally inducible	
lacP$^-$	inefficient binding of RNA polymerase	O	O	O	P^+Z^+/P^-Z^-	inducible at maximum rate	P^+ is *cis* dominant but *trans* recessive
	or	I+	I+	I+	P^+Z^-/P^-Z^+	inducible but at a reduced level	

The activity of the *lac* structural genes is expressed as: I++ strongly inducible; I+ weakly inducible; C constitutive; O no activity.

Mutations in the Operator Region

The operator gene, *O*, is a nucleotide sequence located at the proximal end of the *Z* gene and close to the point at which transcription starts, and it is the sequence to which the repressor binds so as to switch off the *lac* structural genes. The active wild-type operator, O^+, mutates to inactive forms, O^c, which have nucleotide sequences that can no longer bind the repressor so that the *Z*, *Y* and *A* proteins are synthesised constitutively (figure 15.7c). There are at least twelve different sites within the operator region at which mutation can give rise to an operator constitutive phenotype.

At first it was thought that the operator was not only sensitive to the repressor but was also the initiation point for the transcription of the *Z*, *Y* and *A* structural genes, so that in effect the operator was also the beginning of the *lacZ* gene. However, it was soon found that operator constitutive strains produced a wild-type β-galactosidase, in spite of the fact that some were the result of deletions involving all or most of the operator region; *O* and *Z* are almost certainly separate genetic entities.

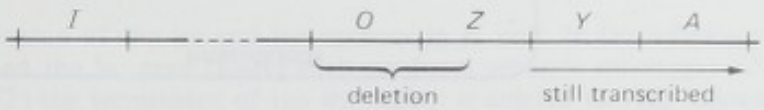
Mutations within either the operator gene or the *lacI* gene can produce constitutive strains, but the O^c and I^- mutations can be distinguished because not only do they map at different (although very closely linked) positions within the *lac* operon but they also behave differently in partial diploids. We have already seen that I^-Z^+/I^+Z^+ partial diploids are inducible, but O^cZ^+/O^+Z^+ strains are constitutive; whereas I^+ is dominant over I^-, O^+ is recessive to O^c. Furthermore, although both I^+Z^-/I^-Z^+ and I^+Z^+/I^-Z^- are inducible, so that it does not matter whether or not I^+ and Z^+ are on the same or different molecules of DNA, a very different situation exists with the O^c mutations. In O^cZ^+/O^+Z^- (*cis*) partial diploids Z^+ is expressed constitutively but in O^cZ^-/O^+Z^+ (*trans*) strains Z^+ activity is inducible; thus only those genes on the same molecule of DNA as O^c are expressed constitutively and although O^c is dominant to O^+ in the *cis* position it is recessive to O^+ in the *trans*. These results are entirely consistent with the idea that the diffusible repressor specified by *lacI* recognises and binds to the wild-type operator switching off those genes to which the operator is adjacent.

When it was first suggested that the operator, in addition to being the recognition site of the repressor, might also initiate transcription, Jacob and Monod predicted that operator-negative (O^o) mutants might be found which were unable to initiate transcription and so could not produce any of the *lac* proteins. When mutants lacking all the *lac* proteins were found, the mutations mapped at the extreme proximal end of *lacZ* and they appeared to correspond to the operator mutations predicted by Jacob and Monod. However, it was subsequently found that the activity of these mutants could be restored to normal by nonsense suppressor mutations, which enable nonsense triplets to be read as sense so that chain elongation can occur across a chain-termination triplet (page 299); thus the so-called O^o mutations are no more than extreme polar mutations at the proximal end of *lacZ*. No true operator-negative mutants have been found, because transcription is *not* initiated from the operator but from another distinct nucleotide sequence, the promoter.

The Promoter

The promoter region (*P*) was first identified in 1966 following the discovery of mutant strains which although inducible had a greatly reduced maximum rate of enzyme synthesis—that is to say that although the mechanism of regulation was not affected and the operator was demonstrably O^+, these mutants transcribed the *lac* genes at a greatly reduced rate. The promoter initiates transcription by providing a site at which the molecules of RNA polymerase can bind and so can only initiate the transcription of genes on the same molecule of DNA; because of this promoter mutations, like O^c mutations, are *cis* dominant and *trans* recessive (P^+Z^+/P^-Z^- partial diploids are inducible and produce *lac* proteins at the normal rate, but P^+Z^-/P^-Z^+ strains have reduced maximum rates of *lac* protein synthesis).

The promoter was inferred to be located between *lacI* and the operator because deletions involving the operator and extending into *lacZ* still produce the *Y* and *A* proteins, showing that *lacY* and *lacA* were still being transcribed and that the initiation site could not be between *O* and *Z*. Deletion mapping has confirmed that the promotor is between *I* and *O*.



The Terminator

It so happens that the *I* gene on the one hand and the *Z*, *Y*, and *A* genes on the other are transcribed in the same direction, so that the *I* gene mRNA and the *Z-Y-A* gene mRNA must be templated along the same strand of DNA, and yet transcription normally stops at the end of *I* and does not continue on to transcribe *Z*, *Y* and *A* on to the same molecule of mRNA; likewise, transcription of the *Z-Y-A* gene cluster terminates near the end of *lacA*. The existence of a specific terminator was first shown using strains of *E. coli* carrying the L1 deletion; these strains lack part of *I*, and part of the promoter and transcription of *I* continues into the *Z-Y-A* region, suggesting that the L1 deletion has removed the termination signal at the end of the *I* gene—this *read through* is greatly reduced if an F-prime factor carrying I^+ is also present, as this specifies an active repressor which attaches to the *lac* operator and prevents transcriptional read through (figure 15.8).

An interesting confirmation of this and other aspects of the operator-repressor hypothesis comes from the work of William Reznikoff and his colleagues; by using a clever series of tricks they were able to join all or part of the *lac* region on to the distal part of the tryptophan operon and so to bring any remaining *lac* genes under the control of the tryptophan regulatory system. In these fusion strains transcription commenced at the operator end of the *trp* region and, because the tryptophan region terminator had been deleted, continued on into *lacI* (figure 15.9). If the *lac* operon was intact, the *lacI* terminator stopped transcription and the *Z*, *Y* and *A* genes were not transcribed,

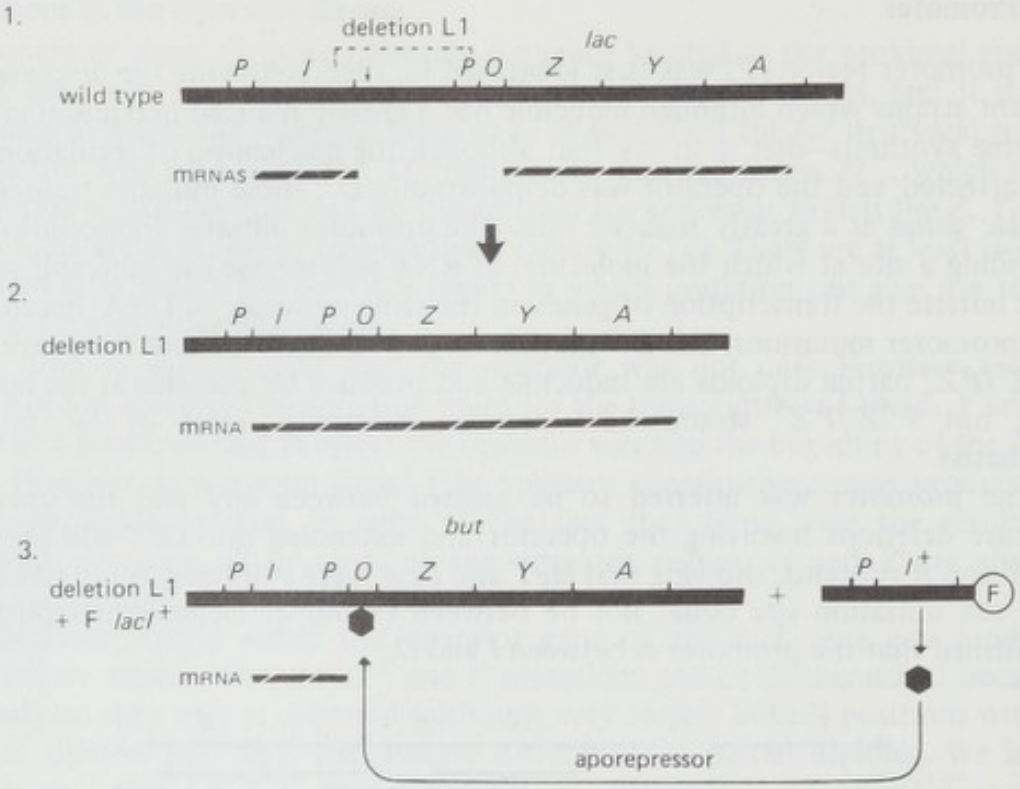


Figure 15.8 The terminator. In the wild-type (1) the *I* and *Z–Y–A* genes are transcribed independently, but when the L1 deletion is present (2) transcription of *I* continues through the *Z–Y–A* region suggesting that the deletion has removed a specific termination site (small arrow) at the distal end of *I*.

When an I^+ gene is present on an F factor (3) the wild-type repressor is present; this binds to *O* and prevents transcriptional read-through into the *Z–Y–A* region.

but if the distal end of *lacI* was missing, as when the L1 deletion was also present, this terminator was missing and transcription continued on into the *Z*, *Y* and *A* genes, eventually resulting in the production of *lac* proteins. Whenever the *trp* genes were switched on the *lac* genes were also switched on, and vice versa, so that the expression of the *lac* genes was determined by the expression of the *trp* operon.

The *lac* Repressor

The most direct confirmation of the operator-repressor hypothesis followed the isolation of the *lac* repressor by Walter Gilbert and Benno Müller-Hill in 1966. The *lacI* gene is not under the control of an operator-regulator mechanism and the repressor is synthesised constitutively, and in a wild-type cell containing a single I^+ gene there are fewer than twenty repressor molecules (about 0.002 per cent of the cellular protein), each a tetramer (M.W. 150 000) of four identical subunits. The repressor tetramer specifically binds to *lac* region DNA, but *only* if it contains a wild-type operator (O^+), not only identifying the operator as the target site for repressor action but also demonstrating that this control

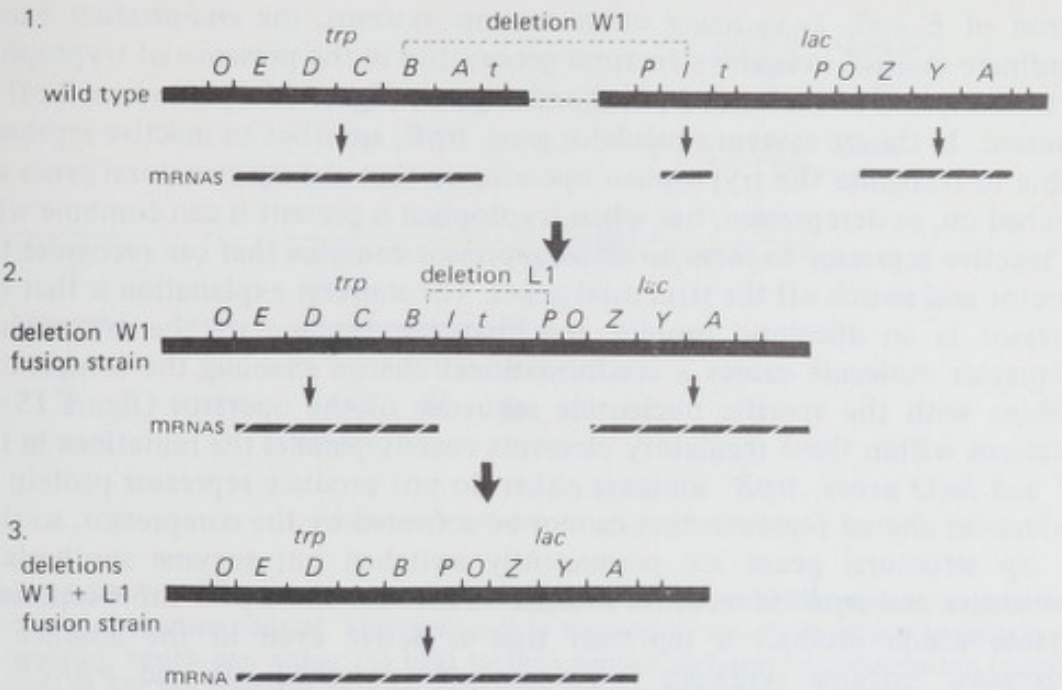


Figure 15.9 Fusion of the *lac* and *trp* operons in *E. coli*. In the wild-type (1) the *trp* gene cluster, *lacI* and the *lac* gene cluster each produces separate messenger RNA molecules. In deletion W1 (2) the terminator of the *trp* region is deleted so that transcription continues until it reaches the *lacI* terminator. The *lac* cluster is transcribed independently. When deletion Ll is also present (3) the *lacI* terminator is deleted and transcription continues from the *trp* genes until it reaches the terminator at the end of *lacA*.

mechanism operates at the level of transcription rather than at the level of translation. The binding of the repressor to the operator DNA does not occur if coinducer is also present, as this can bind to the repressor and prevent its attaching to the operator; these effects are most readily explained if the repressor is an allosteric protein with binding sites for both the operator and the coinducer (the concept of allostery is illustrated in figure 15.2).

The size of the repressor is such that it can only bind to a few turns of the double helix of DNA, suggesting that the length of the operator region is not more than fifty–sixty nucleotide pairs; the operator cannot be less than twelve nucleotide pairs long as this is the minimum number of mutational sites already identified within the region, and if it were shorter there would be a high probability that the same nucleotide sequence would be found by chance outside the operator region, and too many incorrect bindings would occur.

Other Regulatory Systems

The Tryptophan Operon

In the *lac* system the *substrate* acts as an *inducer* by combining with the repressor and preventing the transcription of messenger, but in the tryptophan

operon of *E. coli*, as in many other enzyme systems, the *end-product* exerts coordinate control over the structural genes; thus in the presence of tryptophan the transcription of the *trpE, D, C, B* and *A* genes (figure 15.3) is switched off or *repressed*. In the *trp* system a regulator gene, *trpR*, specifies an inactive repressor unable to recognise the tryptophan operator so that the *trp* structural genes are switched on, or derepressed, but when tryptophan is present it can combine with the inactive repressor to form an active repressor complex that can recognise the operator and switch off the structural genes. The simplest explanation is that the repressor is an allosteric protein and that the binding of the tryptophan corepressor molecule causes a conformational change enabling the complex to combine with the specific nucleotide sequence of the operator (figure 15.4). Mutations within these regulatory elements exactly parallel the mutations in the *lacI* and *lacO* genes. $trpR^-$ mutants either do not produce repressor protein or produce an altered repressor that cannot be activated by the corepressor, so that the *trp* structural genes are permanently switched on; enzyme synthesis is constitutive and $trpR^-$ is recessive to $trpR^+$. There are also trp^{SR} super-repressed mutants which produce a repressor that is active even in the absence of corepressor; enzyme synthesis is permanently switched off and $trpR^{SR}$ is dominant to $trpR^+$. $trpO^c$ operator mutants, as in the *lac* system, have lost their ability to combine with the repressor and are constitutive.

The repressible enzyme systems are those involved in anabolic processes whereas inducible systems are generally those involved in catabolism.

The *lac* and the *trp* systems are both examples of *negative* control as in the *absence* of any cytoplasmic repressor the operon is switched *on*, but other systems are under *positive* control and the operon is only switched on by the interaction of the operator with the product of the regulator gene, in positive control systems sometimes called an *apo-inducer*. Positive control systems, like negative control systems, can be either inducible or repressible.

The Arabinose Operon in *E. coli*

One of the best-known examples of a system involving positive control is the regulation of the l-arabinose pathway in *E. coli*, of particular interest because it has features of both positive and negative control. The system for arabinose metabolism consists of three closely linked genes (*araD, B* and *A*) specifying the three enzymes required to convert L-arabinose to D-xylulose-5-phosphate, a regulator gene (*araC*), operator and initiator genes (*araO* and *araI*) and an unlinked structural gene (*araE*) specifying a permease (figure 15.10). The system is inducible and on the addition of arabinose inducer, the amounts of all the enzymes are coordinately increased. The three genes *araD, A* and *B* are transcribed from *araB* to *araD* on to a molecule of polygenic messenger.

The $araC^+$ gene specifies a protein which binds specifically to DNA from the *ara* region and $araC^-$ mutants lack this protein and have lost the ability to synthesise any of the enzymes, including the permease specified by the unlinked *araE* gene. The $araC^-$ mutants are uninducible but since C^-B^+/C^+B^- and C^-B^-/C^+B^+ partial diploids can be induced $araC^-$ is *recessive* to $araC^+$; this is in

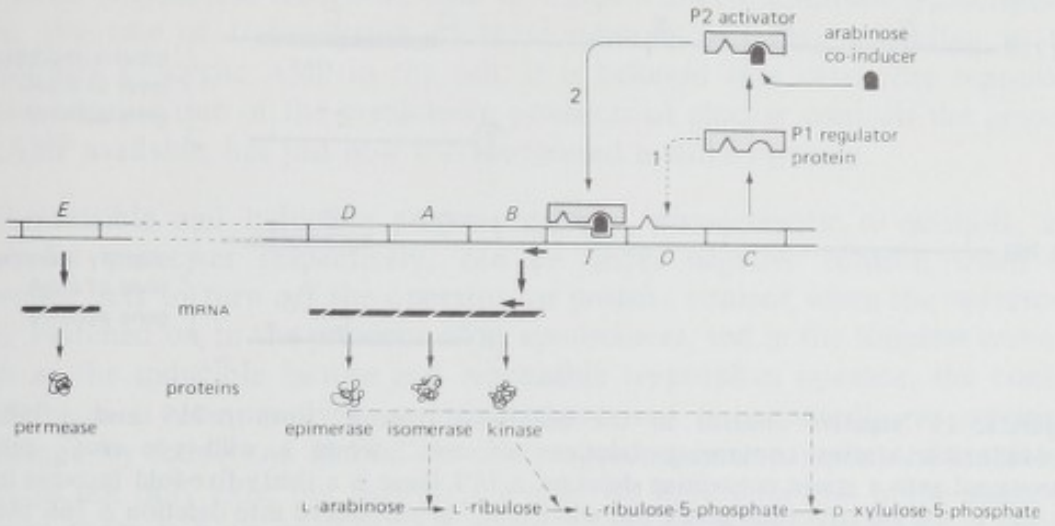


Figure 15.10 The arabinose operon and its regulation. The arabinose operon is under both positive and negative control. The *araC* gene is thought to specify an allosteric protein (the P1 protein), which can either (1) bind to the operator and repress transcription (negative control) or (2) can combine with an arabinose co-inducer to form the P2 activator protein, which then binds to an initiator sequence (*I*) adjacent to *araB* and stimulates transcription (positive control).

contrast to the uninducible *lacI*^{s} mutations which are *dominant* to *lacI*$^{+}$. Clearly *araC* is neither an operator nor promoter and the probable explanation is that *araC* specifies an allosteric regulator protein (P1) which is activated by combining with l-arabinose coinducer to form an activator complex (P2); transcription can only be initiated when the P2 activator complex binds to a recognition sequence adjacent to *araB*, so that in the absence of either P1 protein or coinducer the synthesis of mRNA cannot normally occur.

Other *araC* mutants (*araC*^{c}) are constitutive and are *dominant* to *araC*$^{+}$, again in contrast to the constitutive *lacI*$^{-}$ mutations which are *recessive* to *lacI*$^{+}$. The simplest explanation of these is that *araC*^{c} produces an altered repressor protein which can activate the operon without the intervention of coinducer.

The target site for the P2 activator is a nucleotide sequence between *araC* and *araB* called the initiator (*araI*). This region was first identified because the Ara^{-} deletion mutant, Δ719, which appears to be missing all of *araC*, reverts to strains (*araI*^{c} *araC*$^{-}$) which produce the *ara* enzymes constitutively, although at a rather low level; these reversions still contain deletion Δ719 and in addition a mutation between *araC* and *araB*. Note that the *araC*$^{+}$ regulator protein is still able to activate *araI*^{c} as *araI*^{c} *araC*$^{+}$ strains have much higher basal levels of enzyme synthesis than have *araI*^{c} *araC*$^{-}$ strains. Thus even in the absence of inducer the regulator protein can cause an increase in the expression of the *ara* structural genes. The initiator region is clearly a target site for positive control and so is not an operator in the way envisaged by Jacob and Monod.

The nature of the *araC* and *araI* mutations shows that the regulator protein plays a positive role in enzyme induction; the following experiments carried out by Ellis Englesberg show that this regulator protein also plays a negative role by attaching to the operator and preventing transcription. The deletion strain Δ719

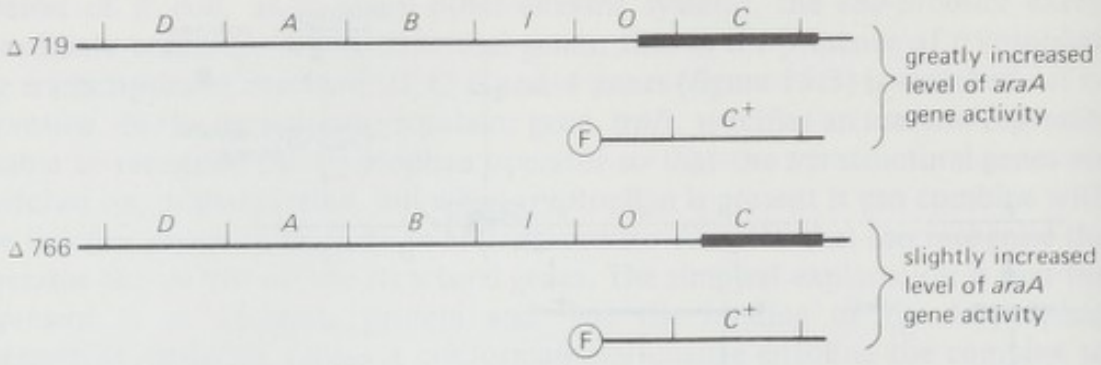


Figure 15.11 Negative control in the arabinose operon. Both Δ 719 and Δ 766 are non-inducible strains containing deletions of *araC*. When a wild-type *araC*$^+$ gene is introduced into a strain containing deletion Δ 719 there is a thirty-five-fold increase in the activity of the *ara* structural genes, but when *araC*$^+$ is introduced into deletion Δ 766 there is only a two-fold increase. The probable explanation is that in Δ 719 the operator has been deleted so that the only effect of the regulator protein is to activate the structural genes. Δ 766 still contains the operator and so can be negatively repressed by the regulator protein.

appears to be missing not only all of *araC*, and so behaves as a non-inducible *araC*$^-$ mutant, but also part of the region between *araC* and *araB* (figure 15.11). When an F-prime factor carrying *araC*$^+$ was introduced into Δ719 there was, even in the absence of inducer, a 35-fold increase in the amount of *araA* protein present. When the experiment was repeated using a different mutant, Δ766, where the deletion is confined to the *araC* gene, there was only a two-fold increase in the amount of *araA* protein. Thus in Δ719 the wild type *araC*$^+$ gene product causes a much greater increase in the expression of *araA* than in Δ766, yet the only difference between these *araC* deletion mutants is that in Δ719 part of the region between *araC* and *araB* is also missing. The probable explanation is that Δ719 is lacking an operator region which is present in Δ766—thus in Δ766 the P1 regulator protein can attach to the operator and play a negative repressive role by preventing transcription, while in Δ719 the operator is deleted and so the introduction of the regulator protein can only result in activation. This model for the control of the arabinose operon is summarised in figure 15.10.

Catabolite Repression

Yet another form of control acts at the level of transcription. Many enzymes are involved in the utilisation of glucose and if *E. coli* is grown in the presence of glucose the production of these enzymes is either switched off or turned down—thus, in the presence of glucose, the activity of the *lac*, *gal* and *ara* operons is switched off. This phenomenon is called glucose inhibition and efficient transcription is prevented by a control process called *catabolite repression* which, unlike the operator–regulator system, acts via the promoter.

In these operons that are sensitive to glucose inhibition it appears that RNA polymerase does not bind directly to the promoters (or else binds very inefficiently) unless (i) a special protein factor called CAP binds to 3′–5′ cyclic AMP (cAMP) and (ii) the CAP-cAMP complex binds to the promoter; only now

can RNA polymerase recognise, bind to the promoter and initiate transcription. Thus the rate of transcription of these operons will vary according to the availability of cyclic AMP in the cell; it is believed that catabolite repression occurs because one of the breakdown products of glucose controls the amount of cAMP available, but just how this is achieved is still a mystery.

Repressible and inducible enzyme systems, characteristic of anabolic and catabolic processes respectively, can be under negative control, when the *repressor* acts to turn *off* the operator, or positive control, when the operator is only switched *on* in the presence of an apo-inducer, and in the simplest systems, such as the inducible lactose and repressible tryptophan operons, the control mechanism is comparatively straightforward and is, essentially, as originally envisaged by Jacob and Monod. In other operons more complicated regulatory systems not only exist but may be the rule. We have seen that in the arabinose operon there is both positive and negative control. In the repressible histidine operon of *Salmonella typhimurium* the nine structural genes are under the control of a single operator (figure 15.1) and histidine constitutive phenotypes can be produced by mutations at at least five other loci; two of these, *hisS* and *hisR* probably code for His-tRNA synthetase and $tRNA^{His}$ respectively, suggesting that the active repressor is a complex of repressor and His-$tRNA^{His}$. The arginine control system in *E. coli* consists of nine structural genes located at six different positions on the bacterial chromosome, and although physically separated these genes are all coordinately repressed by the same species of repressor when arginine corepressor is present; this shows that it is not necessary for related genes to be contiguous to be under the same control system, and each locus or group of loci presumably has a copy of the operator on which the same repressor can act. Although not unexpected this is an important finding because in higher organisms, with the exception of some fungi, there is little or no tendency for genes of related function to be contiguous, or indeed even on the same chromosome, as they are in bacteria, and yet it is quite clear that the expression of these genes must be under some system of coordinate regulation. So far there is no experimental evidence that operons exist apart from those of viruses, bacteria and fungi but it is possible that their existence will be revealed as more refined methods become available for the genetic and biochemical study of animal cells in tissue culture.

Summary of Key Points

(1) Feedback inhibition, where the first enzyme in a biosynthetic pathway is reversibly inactivated by combining with the end product of the pathway, regulates the *rate* of synthesis of many compounds.

(2) A coarse control, which *coordinately* regulates the production of the enzymes themselves, is achieved by induction and repression.

(3) In inducible systems the enzymes are only produced in the presence of the substrate while in repressible systems the production of the enzymes is repressed in the presence of the end product.

(4) In microbial systems the following components of control systems have been identified:

(i) the structural genes, which encode the enzymes and whose activity is regulated;
(ii) a regulator gene, which produces a specific repressor protein;
(iii) an operator region (gene) to which the repressor can attach so switching off the structural genes;
(iv) a promoter region (gene) which binds the RNA molecule necessary to initiate transcription.

(5) In inducible systems the substrate induces gene activity by binding to and inactivating the repressor so switching on the structural genes. In repressible systems the repressor cannot combine with the operator until it is activated by binding with the end product.

(6) Mutations within all these genes have been identified in the *lac* system of *E. coli.*

(7) In many systems regulation is more complex and incorporates features of both positive and negative regulation.

(8) In higher organisms genes of related function are not grouped together as they are in procaryotes and it is still a matter of opinion as to whether similar systems of control exist.

16 Recombination

They are ill discoverers who think there is no land when they can see nothing but sea.

Francis Bacon (1561–1626)

Although mutation, the process by which one gene suddenly changes into another, is the ultimate source of all new genetic variation, recombination or crossing-over is the fundamental phenomenon of genetics. Apart from its uses as the main tool of genetic analysis it is of great practical importance, as by using genetic recombination the plant or animal breeder endeavours to combine into a single genotype all the most desirable genes; in nature, in just the same way, recombination gives rise to some particularly favourable gene combinations which produce superior phenotypes favoured by natural selection, and other less favourable gene combinations which produce inferior individuals that tend to be eliminated from the population.

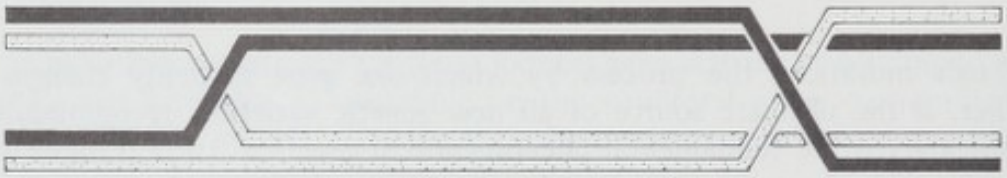
Even though recombination is of such fundamental importance and has been more studied than almost any other aspect of genetics, it is still one of the least understood phenomena of genetics, and only within the last few years have we obtained even the most superficial understanding of the molecular events that take place during recombination.

The Chiasmatype Theory

The first theory of recombination appeared in 1909 in an important paper by the Belgian cytologist F. A. Janssens. He had been studying the cytology of meiosis in Salamanders and was struck by two facts: firstly that there were *two* meiotic divisions, and secondly that at mid-prophase, when the maternal and paternal homologous chromosomes have paired along their lengths and started to contract, the chromosomes divided into chromatids and formed chiasmata (chapter 4). Janssens thought that each chiasma marked a point at which the maternal and paternal chromatids had made contact, broken and rejoined in a new combination, the other two chromatids remaining intact (figure 16.1a); thus each chiasma was the visible manifestation of a recombination event (or *crossing-over* as it was later termed by Thomas Hunt Morgan). According to the chiasmatype theory, each of the four products of meiosis carried different combinations of paternal and maternal chromosome segments, so that two divisions were necessary for these different chromosomes to segregate into the gametes—as we would now say, meiosis is a mechanism for the production of four haploid nuclei of different genotypes.

It must be remembered that Janssen's chiasmatype theory preceded the understanding of linkage, and in 1911 Morgan was able to apply this theory to

(a) the chiasmatype theory



(b) the classical theory

Figure 16.1 Chiasmata.

(a) According to Janssens' chiasmatype theory, each chiasma arises because of the breakage of two non-sister chromatids and their rejoining in a new combination during the four-strand stage of meiosis.

(b) Most cytologists preferred the classical theory, which regarded a chiasma as a point at which there was a change of pairing partner without breakage and reunion.

his results with *Drosophila* and realised that linkage occurred because the genes involved were on the same chromosome. Although the chiasmatype theory was generally accepted by geneticists, it continued to be rejected by the cytologists, who were unable to find any evidence for the breakage and reunion required by the theory and could not envisage how this could happen at a stage when the chromosomes were only loosely coiled around each other and in a contracted state. Their interpretation of chiasmata was that they simply represented a change of pairing partner without either chromosome breakage or recombination (figure 16.1b). This dilemma was only resolved in 1928, when John Belling proposed that the exchanges took place at an earlier stage when the homologous chromosomes were more closely associated and less contracted; thus, while the chiasmata do not show the process of crossing-over, they are the direct consequence of these exchanges.

By 1930 there was overwhelming evidence for a connection between chiasmata and crossing-over, although one objection remained– that the number of chiasmata decreased as metaphase I approached–and so it was thought that chiasmata could not be the manifestation of crossing-over. This objection was removed in 1931 when Cyril D. Darlington showed that this reduction in chiasmata numbers was due to *terminalisation*, when all the chiasmata are pushed together at the extreme ends of the chromosomes (figure 16.2).

Breakage and Reunion Models

During the early 1930s, Darlington developed an intricate model to explain not only recombination but also the movements of the chromosomes during meiosis.

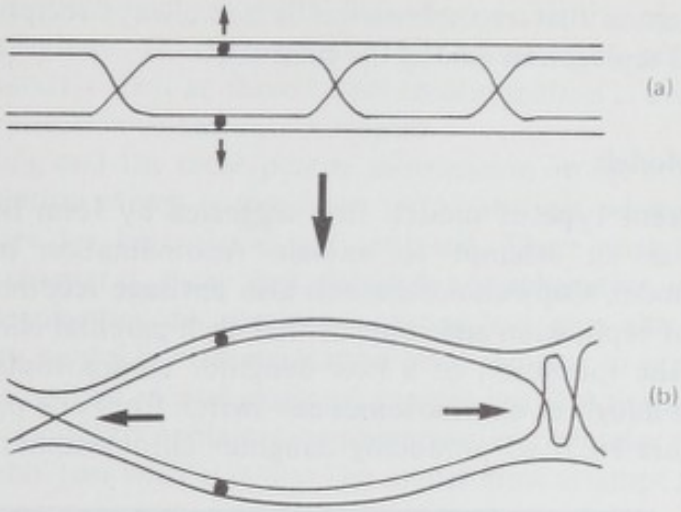


Figure 16.2 Terminalisation.

(a) A bivalent at mid-prophase with three chiasmata. Each chiasma is individually distinguishable under the microscope.

(b) As prophase proceeds the centromeres repel each other, the centromeres are pushed farther and farther apart and the chiasmata are slid towards the ends of the bivalent. Although there are still two chiasmata in the right arm of the bivalent, these are so close together that they appear as one.

This model was partly based on the observation that two members of a pair of homologous chromosomes will attract each other and synapse at the onset of prophase, but that when each chromosome divides into two chromatids the two pairs of chromatids repel each other. He envisaged that during synapsis the two homologues become relationally coiled around each other, in just the same way that two pieces of twisted string, placed side by side and suddenly released; in untwisting themselves they twist around each other until the internal and relational torsions are in equilibrium. Immediately after synapsis there is equilibrium, but when the chromosomes divide into chromatids the attractions between the homologues are replaced by repulsions and the equilibrium is upset and is only restored by the breakage of two non-sister chromatids at precisely corresponding points; the broken ends uncoil by rotating around the unbroken chromatids and in doing so will come into contact with the corresponding broken ends of the non-sisters, and so can rejoin in a new combination. According to this theory chromatid breakage is caused by changes in the torsions within the bivalent brought about by the replication of the chromosomes into chromatids; chromatid breakage releases these torsions and restores equilibrium. Note that since it is the broken (recombinant) chromatids that unwind it is the unbroken chromatids that cross-over to form the chiasma; this is in contrast to the chiasmatype model where the recombinant chromatids cross-over and form the chiasmata.

Both the chiasmatype and Darlington's theories involve the *breakage and reunion* of chromatids and it is a necessary consequence of these models that recombination is always reciprocal and that for each heterozygous marker two of the meiotic products carry one allele and two the other. A serious objection

to these models is that recombination is not always reciprocal and does not always give 2:2 segregation among the products.

Copy-Choice Models

A rather different type of model, first suggested by John Belling in 1931 and later revived in an attempt to explain recombination in bacteria, is the *copy-choice* model. Copy-choice models also envisage recombination as a direct consequence of replication and suppose that each parental chromosome acts as a template for the formation of a new daughter chromosome, and that during replication the daughter chromosomes can switch from one parental template to the other (figure 16.3) so producing daughter chromosomes in part templated

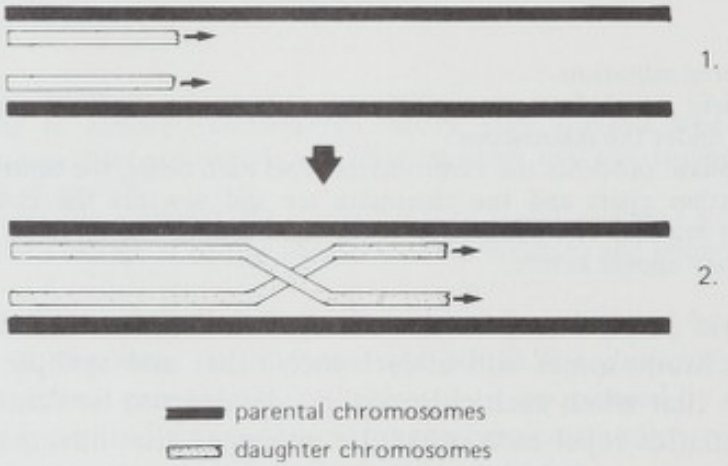


Figure 16.3 Copy choice recombination. Each member of a pair of homologous chromosomes acts as a template for the production of a new daughter chromosome (1). As replication proceeds the daughter chromosomes can switch from one parental template to the other, producing two recombinant and two parental type chromosomes (2).

Note that the two parental chromosomes are preserved intact, so that four-strand crossing-over is not possible.

against the maternal and in part against the paternal chromosomes. These models are unacceptable to geneticists because only one of each pair of sister chromatids could participate in crossing-over as the other chromatids are the original parent chromosomes and therefore non-recombinant, and yet we know from tetrad analysis that three-strand and four-strand double cross-overs occur regularly. Even more serious objections are seen when the copy choice model is translated into molecular terms; not only does it require the conservative replication of DNA, whereas we can be quite confident that DNA replicates semi-conservatively (see chapter 2), but the recombinant chromatids must contain all new and no parental DNA, and again we know that this is not so (see chapter 4).

Finally, although the chromosomes are not visibly divided until mid-prophase, we know from the DNA content of the cells that the chromosomes in fact replicate during the preceding interphase. If we accept that synapsis is a prerequisite for recombination and that chiasmata are the visible consequences

of crossing-over, it is clear that recombination must occur during prophase and so must be independent of replication. This observation strengthens our arguments against models such as these where recombination is dependent upon the simultaneous replication of the chromosomes.

The most revealing and the most precise information on the consequences of individual recombination events comes from tetrad analysis, where the products of individual meioses can be isolated and analysed. These analyses, similar to those presented in chapter 6, show that although recombination is a process of great precision, irregularities do sometimes occur and give rise to abnormal segregations and any model for recombination must attempt to explain not only the precision of the normal recombinational process but also these irregularities. Before discussing a model to explain recombination in molecular terms we shall consider in more detail just what it is that the model must attempt to explain.

Recombination as a Regular Process

The genetic analysis of many thousands of tetrads has revealed three features of the recombinational process. Firstly in the overwhelming majority of the tetrads recombination is reciprocal and there is regular 2:2 segregation for every heterozygous marker (tetrad analysis is described in chapter 6). Secondly, any one recombination event involves only two of the four products of meiosis (and so only two of the four chromatids) and these products carry correspondingly exchanged segments. Thirdly, multiple recombination events may involve two, three, or all four chromatids.

A model for recombination must also be able to account for *high negative interference* (HNI). If *a, b* and *c* are three very closely linked mutations and the frequency of recombination between *a* and *b* is *p* and that between *b* and *c* is *q*, then if *p* and *q* are independent events we expect a proportion *pq* of the recombinants to show recombination both between *a* and *b* and between *b* and *c*, but if there is interference the occurrence of a cross-over between *a* and *b* increases (negative interference) or decreases (positive interference) the probability of a second cross-over between *b* and *c*. In HNI, the occurrence of a cross-over *greatly increases* the probability of a second cross-over occurring in an adjacent region of the chromosome, so that the frequency of multiple exchanges is higher than expected (figure 16.4). In general, HNI can only be detected between pairs of very closely linked markers and the closer the markers the higher the degree of interference; it appears to be of regular occurrence as it has been observed in all genetic systems where fine structure analysis is possible.

Aberrant Segregations

The first regular occurrence of an abnormal segregation was reported by Carl Lindegren in the late 1940s. For example, he noted that in $A \times a$ crosses (A and a are the mating-type alleles) with *Saccharomyces cerevisiae* some of the asci contained ($3A + 1a$) or ($1A + 3a$) ascospores instead of the expected ($2A + 2a$). This phenomenon he termed *gene conversion.* The importance of this

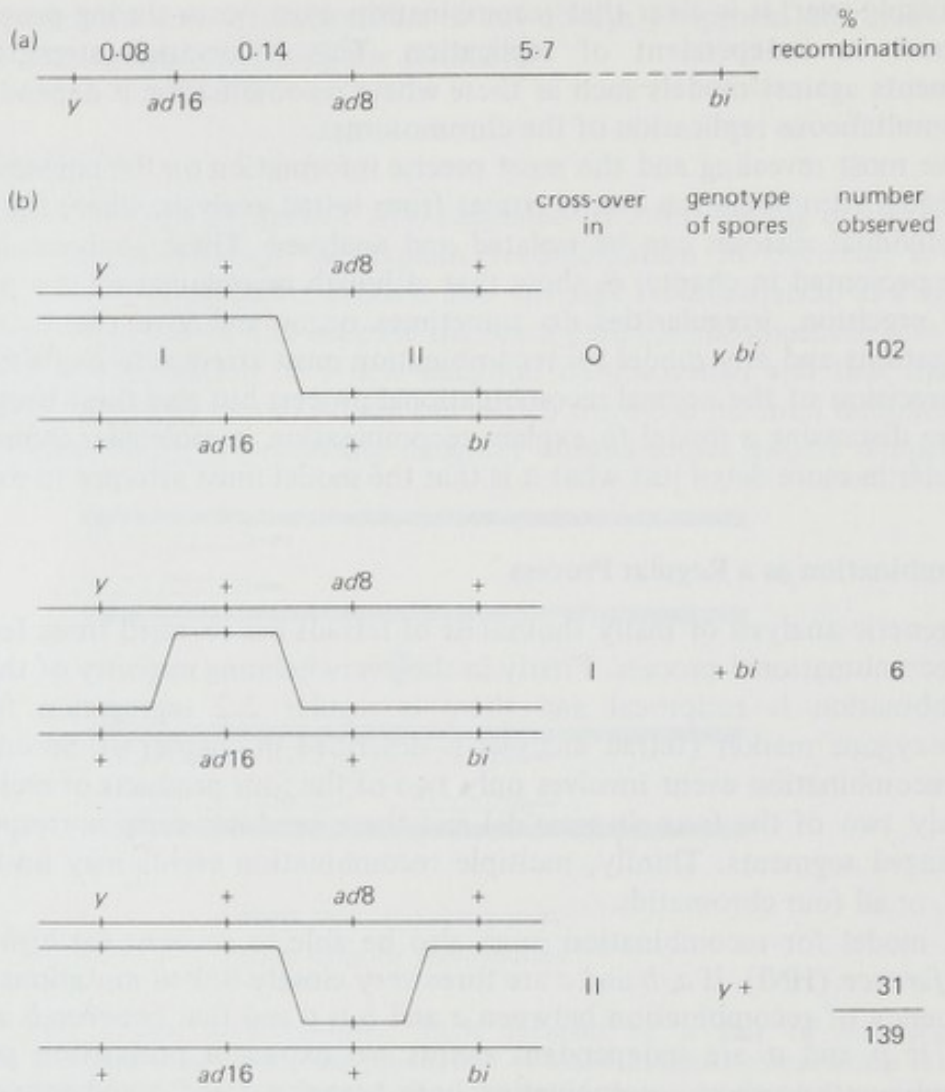


Figure 16.4 High negative interference in *Aspergillus nidulans*.

(a) The linkage map of the *ad8* region. The *y* (yellow conidia instead of green) and *bi* (biotin requirement) loci are very closely linked to the *ad* (adenine requirement) locus.

(b) When Roy Pritchard crossed two allelic *ad* mutants and selected for ad^+ spores (that is, selecting for a cross-over between the mutant *ad* sites), he found that 2.7 per cent ($6/139 \times 100$) were also recombinant between *y* and *ad16*, and 23.4 per cent between *ad8* and *bi*, instead of the expected frequencies of 0.08 and 5.7 per cent respectively. Thus the occurrence of a cross-over between *ad8* and *ad16* has greatly increased the chance of a second cross-over occurring in either region I or region II; there is high negative interference.

unexpected discovery was not realised until 1955, when Mary Mitchell showed not only that similar 3:1 (or 6:2) segregations occurred in crosses between pyridoxine-requiring mutants of *Neurospora crassa* but that a very closely linked site within the same locus still showed normal 2:2 segregation. Mitchell crossed two allelic mutants, designated *pdx* and *pdxp*, and among 585 asci analysed found four which contained two wild-type and six pyridoxine-requiring ascospores. If these had asci arisen by normal reciprocal recombination (figure 16.5), each should have contained two wild-type, two *pdx*, two *pdxp* and

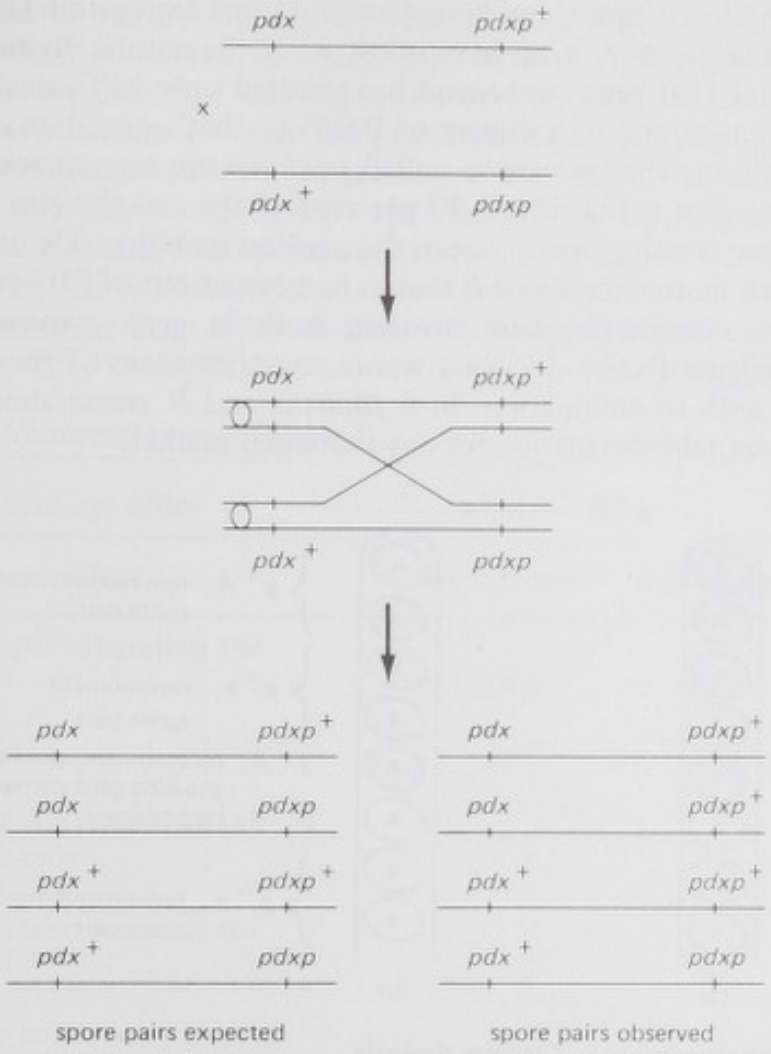


Figure 16.5 Gene conversion in *Neurospora crassa*. When two allelic pyridoxine-requiring mutants are crossed, the asci containing a pair of wild-type spores due to crossing-over between the mutant sites are also expected to contain a pair of the doubly mutant reciprocally recombinant spores; three out of the four asci examined by Mary Mitchell contained instead another *pdx pdxp*$^+$ spore pair. Although there is aberrant 3:1 segregation at the *pdxp* site the very closely linked *pdx* site shows normal 2:2 segregation.

two *pdx pdxp* doubly mutant ascospores, but Mitchell found that three of the four asci did not contain any doubly mutant *pdx pdxp* spores and instead contained four *pdx* and two *pdxp* spores. These results showed that the aberrant segregation was due to the abnormal behaviour ('gene conversion') of a particular mutant site and not to the abnormal behaviour of a whole chromosome.

Lindsay Olive, Arif El-Ani and Yoshiaki Kitani have extensively studied the *g* locus (g^- ascospores are grey instead of black) of *Sordaria fimicola*. Among 200 000 asci examined from $g^+ \times g^-$ crosses, they found 0.06 per cent with 5:3 segregations, 0.05 per cent with 6:2 segregations, and 0.008 per cent with abnormal 4:4 segregations. In the abnormal 4:4 asci there were four g^+ and four g^- spores, but the spores were not arranged in pairs with respect to g^+ and g^-; in

these asci other closely linked markers showed normal segregation (figure 16.6a). In the 5:3 asci segregation must have occurred at the mitotic division following meiosis, implying that gene conversion has affected only *half* a meiotic product (for example, one strand of a duplex of DNA) so that segregation occurs at the succeeding division; this process is called *post-meiotic segregation.* A striking feature of these data is that about 30 per cent of the asci showing 5:3 and 6:2 segregation show crossing-over between the markers on either side of the *g* locus, and what is even more remarkable is that in forty-eight out of fifty-two instances the *same* two chromatids were involved both in gene conversion and in crossing-over (figure 16.6b)—in other words, most instances of gene conversion are associated with recombination. In *S. fimicola* and *N. crassa* about one ascus in 500 shows aberrant segregation for any particular marker.

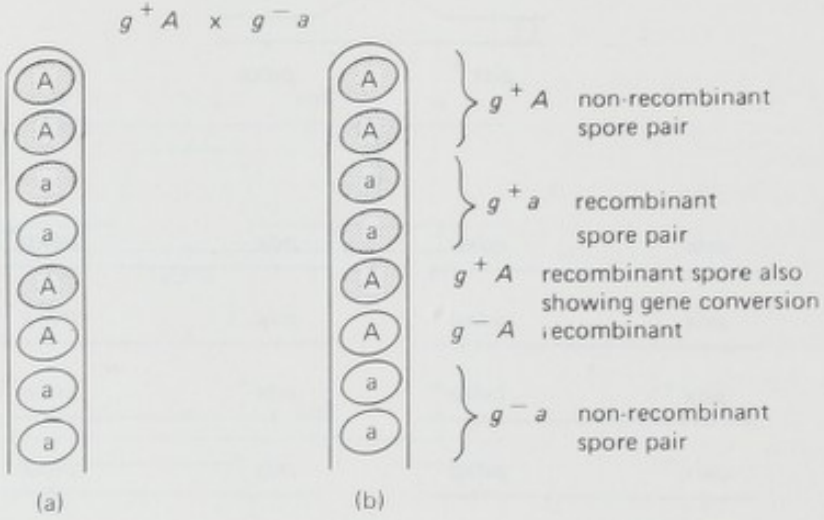
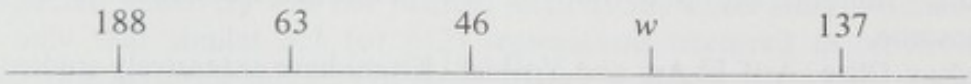


Figure 16.6 Gene conversion in *Sordaria fimicola.*

(a) An ascus showing abnormal 4:4 segregation of g^+ (stippled) and g^- (white) spores together with the normal 4:4 segregation of a closely linked pair of alleles designated *A* and *a.*

(b) An ascus showing 5:3 segregation of g^+:g^- and normal 4:4 segregation of *A*:*a.* The aberrant spore pair shows post-meiotic segregation (gene conversion) of g^+ and g^- and, since one spore is g^-A, is clearly recombinant between *g* and *A.*

A more bizarre situation has been found in the Ascomycete *Ascobolus immersus.* Georges Rizet and Pascal Lissouba studied a series of five allelic mutants having pale ascospores; these they crossed in all pairwise combinations and constructed the linkage map



In these crosses they found some asci where recombination had occurred between the two mutant sites so contained two dark wild-type and six pale ascospores. When the six pale ascospores were tested, none were double mutants, and instead there were two additional spores having the same genotype as the

parent located at the *left* end of the linkage map. For example, in the cross 63 x 46, the asci contained two wild-type, four '63' and two '46' ascospores instead of the expected two wild-type, two '63', two '46' and two '63 + 46'. It is clear that gene conversion may be polarised, so that there is a gradient of conversion frequency running from one end of the gene to the other.

Another interesting experiment was carried out by Seymour Fogel and Robert Mortimer using four allelic arginine-requiring mutants of *Saccharomyces cerevisiae*. The four mutant sites are very closely linked and from other data Fogel and Mortimer were able to estimate the number of base pairs between the mutant sites. They crossed these mutants in pairwise combinations (table 16.1)

Table 16.1 Abnormal segregation in *Saccharomyces cerevisiae*

Linkage order	4 — 1 — 2 — 17		
Cross	*arg*4 x *arg*17	*arg*1 x *arg*2	*arg*2 x *arg*1
Nucleotide pairs separating the mutant sites	1060	520	128
Asci analysed	697	502	544
Asci showing conversion at the proximal site only	8	8	4
Asci showing conversion at the distal site only	38	21	5
Asci showing conversion at both sites	3	23	27
Asci showing abnormal segregations	49	50	36
Asci showing reciprocal recombination	9	5	0

Fogel and Mortimer crossed four allelic arginine-requiring mutants in pairwise combinations and fully analysed all the asci by back-crossing with each of the parental strains. The table shows the numbers of abnormal asci showing gene conversion at the proximal (left hand) site only, the distal site only and at both sites. Note that in these crosses between closely linked sites more asci show gene conversion than show normal reciprocal recombination.

and all the asci were completely analysed by back-crossing each ascospore to both arginine-requiring parents. Their results showed several interesting features. Firstly, the distal site is converted more frequently than the proximal site, confirming that there is a polarity of conversion from one end of the gene to the other. Secondly, they found that some asci showed gene conversion at *both* the sites of heterozygosity, and they noted (i) that the frequency of double

conversions increased as the distance between the mutant sites decreased, and (ii) that the marker combinations in each doubly converted ascus showed that the *same* chromatid was involved in *both* conversions. This clearly shows that conversion involves a *segment* of the chromosome up to about 1000 nucleotide pairs long—thus, when two mutant sites are very closely linked, double site conversions will be the more frequent, while single-site conversion will prevail when the sites are farther apart.

Gene Conversion in *Drosophila*

Gene conversion has also been observed in *Drosophila* and so is not a phenomenon confined to the fungi, and it will probably be detected in other organisms when suitable test systems are developed. In *D. melanogaster* the rosy gene (*ry*), located on the right arm of chromosome III and the structural gene for xanthine dehydrogenase, offers a unique opportunity for fine-structure analysis as homozygous *ry*/*ry* zygotes are unable to hatch on medium supplemented with purines whereas wild-type zygotes (*ry*/*ry*$^+$ and *ry*$^+$/*ry*$^+$) will hatch. This makes it possible to select for the very rare wild-type flies that arise in crosses between two different but allelic rosy mutants (*ry*/*ry* flies have brownish eyes instead of red); the total number of zygotes is estimated from an identical cross on standard medium so that recombination frequencies can be measured and a fine-structure map of the region constructed. Using this system Arthur Chovnick has devised an ingenious method allowing the recovery of half tetrads and the detection of gene conversion. He synthesised strains carrying a compound chromosome III with *two right arms*, these arms carrying different rosy alleles and heterozygous for the flanking markers; these chromosomes can be represented

$$\underline{e\ d\ \ ry^{x}\ \ c\ b\ a} \bullet \underline{A\ B\ C\ \ ry^{y}\ \ D\ E}$$

At meiosis in the females the two homologous arms of this chromosome can pair together and undergo recombination—when the cross-over is between the pair of chromatids shown in figure 16.7a the two recombinant products (that is, the two chromosome arms) will remain together on the same compound chromosome, a half-tetrad. Chovnick made the cross shown and selected exceptional *ry*$^+$ survivors on purine-enriched medium. The compound chromosome III carried by each survivor was analysed by crossing the flies with a suitable tester strain. Of eighteen *ry*$^+$ half tetrads examined, only six resulted from reciprocal crossing-over. The remaining twelve could not be accounted for by a single reciprocal cross-over and were attributed to the conversion of either *ry*^{5} or *ry*^{41} to *ry*$^+$. This conversion was only observed when the compound chromosome was in the female; since crossing-over does not occur in *Drosophila* males, it appeared that gene conversion in *Drosophila*, as in the Ascomycetes, is associated with recombination. Neither could conversion be explained by mutation as the exceptional flies only arose from heterozygous females and not from homozygotes.

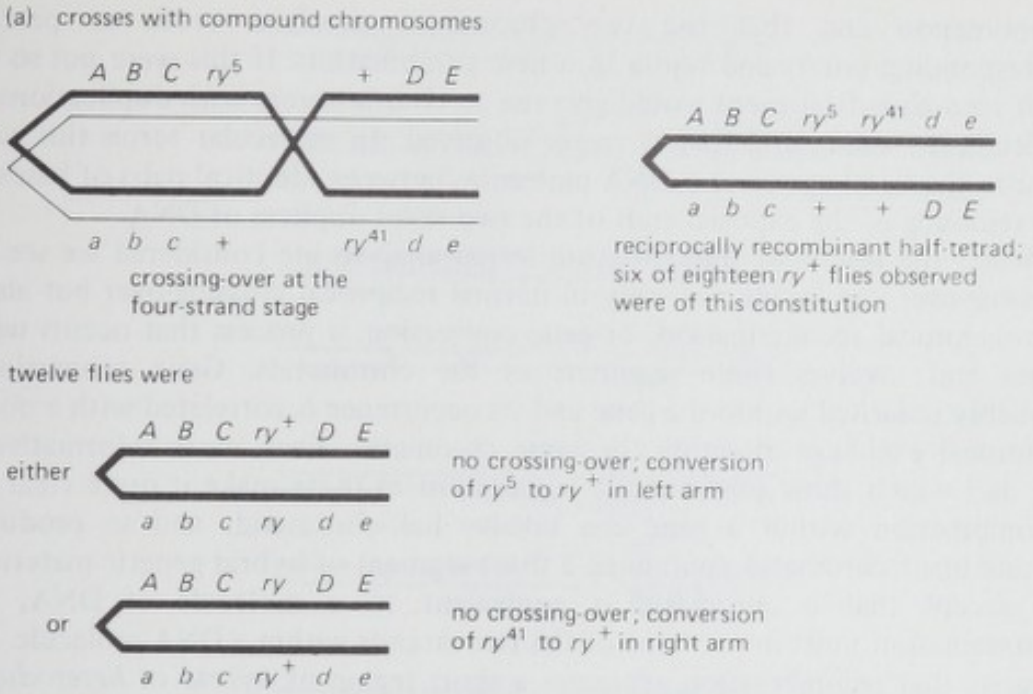


Figure 16.7 Crossing-over and gene conversion in *Drosophila*. Cross (a) uses a female with a compound chromosome III, having two right arms and no left arms; cross (b) uses a female with a normal chromosome III. ry^5 and ry^{41} are allelic rosy mutants and the exceptional ry^+ progeny are selected on purine-enriched medium. In both crosses a homozygous ry/ry male was used as the other parent.

Similar crosses with normal chromosomes III also yielded progeny that could not be accounted for by a single reciprocal cross-over (figure 16.7b).

These results completely confirm the reciprocity of crossing-over between *distantly* linked markers, but for very closely linked markers both reciprocal crossing-over and gene conversion occur and the closer the two *ry* mutant sites the greater the proportion of exceptional progeny due to gene conversion.

The Molecular Basis of Genetic Recombination

The older breakage and reunion theories assumed that recombination is a *point*

phenomenon and that the two chromatids involved break at precisely corresponding points and rejoin in a new combination. If this were not so then each recombination event would give rise to chromosomes with duplications and deficiencies, something that is rarely observed. In molecular terms this would require the breakage of two DNA molecules between identical pairs of bases and the rejoining of the exposed ends of the two sister duplices of DNA.

When the results of fine structure tetrad analysis are considered we see that crossing-over can result not only in normal reciprocal crossing-over but also in non-reciprocal recombination, or gene conversion, a process that occurs *within* genes and involves finite *segments* of the chromatids. Gene conversion is probably polarised within the gene and its occurrence is correlated with a normal reciprocal exchange affecting the same chromatid. Even more informative are the asci which show post-meiotic segregation as these make it quite clear that recombination within a gene can involve half-chromatids and so produce a recombinant chromatid containing a short segment of hybrid genetic material. If we accept that a chromatid is equivalent to a molecule of DNA, then recombination must involve one of the two strands within a DNA molecule. This suggests that recombination generates a short region of *hybrid* or *heteroduplex* DNA, within which one strand of the duplex is of maternal and the other of paternal origin.

The Polaron Hybrid DNA Model for Genetic Recombination

Several models for recombination have been proposed that are based on the concept that recombination involves the formation of short segments of hybrid DNA; they all accept that DNA replication occurs during interphase, that recombination occurs during meiotic prophase when the homologous chromosomes are closely paired, and that recombination and replication are independent events. We shall discuss one of these, the *polaron hybrid* DNA *model*, proposed by Harold Whitehouse and P. J. Hastings in 1965. This model (figure 16.8) makes two assumptions: firstly, that short segments of hybrid DNA are formed by the breakage and localised unwinding (dissociation) of single strands, followed by the annealing of complementary single strands from different DNA molecules; and secondly, that the DNA is organised into segments called *polarons*—each polaron might correspond to a gene, and its ends are marked by *linkage points* (represented by the vertical bars in figure 16.8) where strand breakage occurs and from which strand dissociation commences.

The polaron hybrid DNA model clearly defines recombination as an enzymatic process. Not only is DNA polymerase necessary to synthesise the new complementary strands, but endonucleases and exonucleases are required to nick one strand in each DNA duplex, to dissociate the nicked and unbroken strands, and to break down the old single-stranded DNA.

The two most important features to note are, firstly, that any pairs of linked markers spanning the segment of hybrid DNA will show normal reciprocal recombination; in the cross shown in figure 16.8, each recombinant type tetrad will contain two recombinant (P^-R^+ and P^+R^-) and two non-recombinant

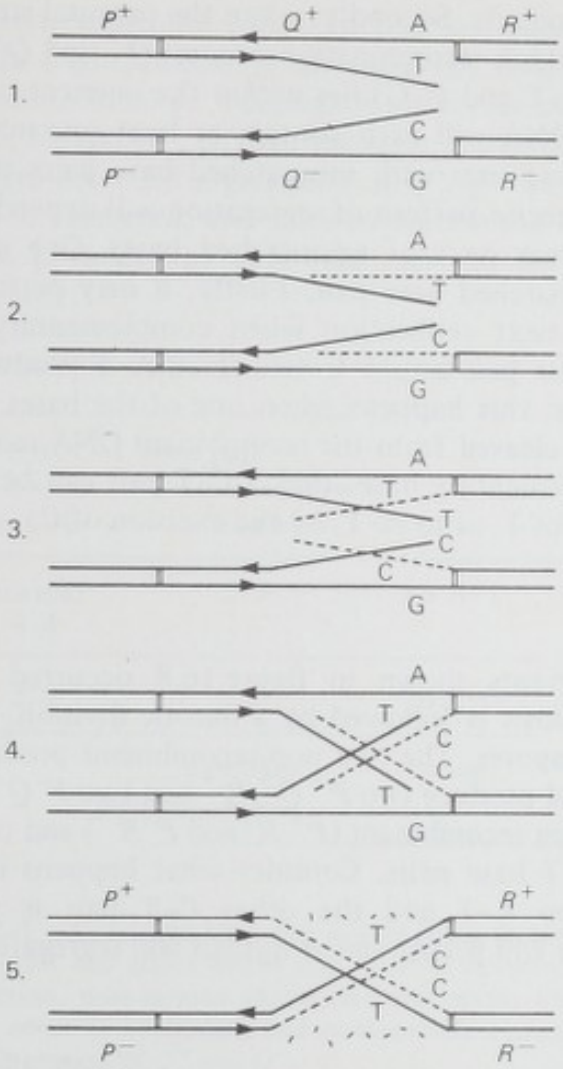


Figure 16.8 The polaron hybrid DNA model for recombination. The diagrams show the outcome of the cross $P^+ Q^+ R^+ \times P^- Q^- R^-$ when crossing-over takes place within the Q gene. Q^-, an allele of Q^+, has a G–C instead of an A–T base pair at one particular site; P and R are closely linked loci on either side of Q. Although each cross-over gives rise to reciprocal recombinants for P^+, P^- and R^+, R^-, the segregation of Q^+ and Q^- will depend on what happens to the mismatched C–T base pairs. Note that crossing-over does not take place at a point but involves homologous lengths of DNA. Each duplex structure represents a molecule of DNA (chromatid) and the vertical bars are the linkage points defining the ends of the polaron. Only the two chromatids taking part in recombination are shown.

(1) The two homologous molecules of DNA (chromatids) are paired together. An endonuclease recognises corresponding linkage points on each molecule and nicks *one* strand in each duplex; this breakage involves strands of *opposite* polarity. The broken strands now uncoil towards the other end of the polaron, so dissociating from the unbroken strands.

(2) DNA polymerase synthesises new nucleotide strands (dashed) from the linkage points and along the unbroken parental strands, so replacing the dissociated strands.

(3) The new strands dissociate from the unbroken parental strands used as templates.

(4) Two regions of hybrid DNA are now formed by complementary base pairing between strands of opposite polarity, the old strand of one parental molecule annealing with the new strand from the other.

(5) The remaining single-stranded regions are enzymatically digested and polynucleotide ligase seals the gaps, so forming two covalently bonded recombinant DNA molcules.

(P^+R^+ and P^-R^-) products. Secondly, when the parental strain is heterozygous for the gene *within* which recombination occurs (Q^+ and Q^-) *and* when the site of heterozygosity (A-T and C-G) lies *within* the segment of hybrid DNA, the segments of hybrid DNA will each contain at least one mismatched base pair (C-T). It is only these sites with mismatched base pairs that can show gene conversion and the precise pattern of segregation will depend on the subsequent behaviour of these two pairs of mismatched bases. One of two things must happen to each mismatched base pair. Firstly, it may persist as a mismatched base pair until the next replication when complementary base pairing will convert each C-T base pair into a C-G and an A-T product. Secondly, *error correction* may occur; this happens when one of the bases of the mismatched pair is enzymatically cleaved from the recombinant DNA molecule and replaced by the correct complementary base—thus a C-T pair can be corrected either to C-G, by the excision of T, or to A-T, by the excision of C.

Error Correction

Suppose that the events shown in figure 16.8 occurred in *Neurospora* or *Sordaria*, so that meiosis is followed by a mitotic division, producing an ascus containing eight ascospores. The two non-recombinant products of meiosis will replicate normally and produce two $P^+Q^+R^+$ and two $P^-Q^-R^-$ spores. The two remaining products are recombinant (P^+R^- and P^-R^+) and contain hybrid DNA with mismatched C-T base pairs. Consider what happens if one of these C-T pairs is corrected to A–T and the other C–T pair is not corrected; the uncorrected C-T pair will persist and at mitosis will segregate one A-T (Q^+) and

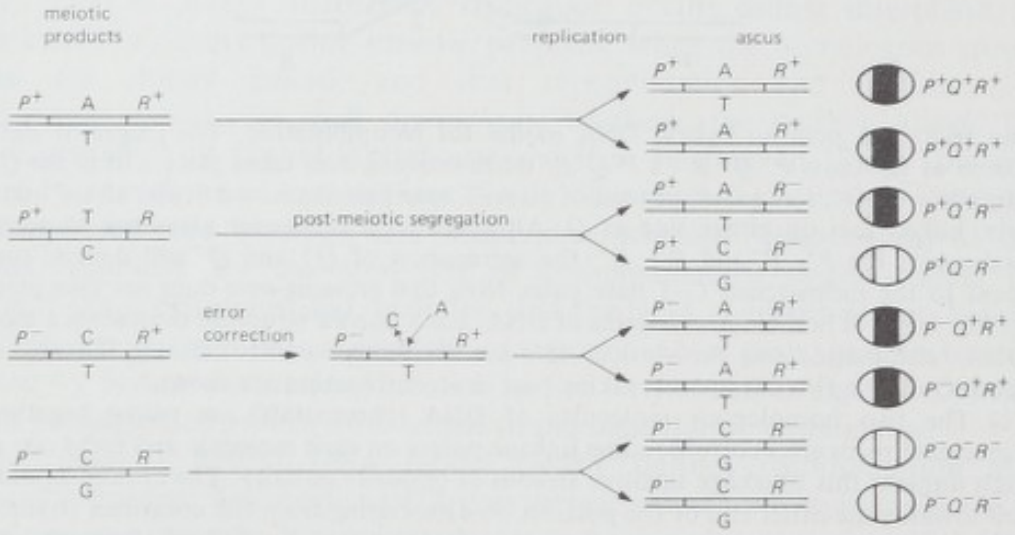


Figure 16.9 Error correction and post-meiotic segregation. At the left are the four products of the meiosis shown in figure 16.8; in many ascomycetes these now undergo mitosis to form an ascus containing four pairs of ascospores (right). One of the mismatched base pairs is not corrected and so undergoes post-meiotic segregation, the other mismatched base pair has been corrected to A–T (that is, to Q^+). Note that in the ascus P^+, P^- and R^+, R^- show normal 4:4 segregation and reciprocal recombination, whereas there is 5:3 segregation of Q^+, Q^-.

one C-G (Q^-) product, while the corrected pair will produce two A-T (Q^+) products. The result (figure 16.9) is an ascus showing 5:3 segregation of $Q^+ : Q^-$ associated with normal 4 : 4 segregation *and* recombination for the pairs of linked markers P^+, P^-, and Q^+, Q^-; had the error correction been towards C-G (Q^-) there would have been 3:5 segregation of $Q^+ : Q^-$. Since neither, either or both C-T pairs can be corrected, and since correction can be in the direction of either A-T or C-G, a variety of abnormal asci can be produced; these are summarised in table 16.2.

Table 16.2 Error correction and post-meiotic segregation

Correction of first C-T base pair				
to A-T	to C-G	none		
6:2	normal 4:4	5:3	to A-T	correction of second C-T base pair
	2:6	3:5	to C-G	
		aberrant 4:4	none	

Error correction of a C-T mismatched base pair can be either towards A-T (wild-type, Q^+) or towards C-G (Q^-), and can affect either one of both mismatched base pairs. In the absence of error correction, post-meiotic segregation will occur. The table shows how the different combinations of error correction and post-meiotic segregation can produce asci showing the aberrant segregation of Q^+ and Q^-.

The polarisation of gene conversion is easily accounted for if the dissociated strands (figure 16.8 (1)), and hence the segments of hybrid DNA, are of variable length as then the further a marker is from the linkage point the *less* frequently will it be included in a segment of hybrid DNA. Since only markers that fall within segments of DNA can show gene conversion there will be a decreasing polarity in conversion frequency from markers near the linkage point to markers at the other end of the polaron. The high negative interference sometimes observed between very closely linked markers can, in part, be explained if the several mutant sites fall within the same segment of hybrid DNA—that is to say, within the same cross-over—as gene conversion can occur simultaneously at each site of heterozygosity.

Although this model does not require the *net* synthesis of DNA, it does involve the limited breakdown and synthesis of DNA and in several organisms there is evidence for a small amount of DNA synthesis during the prophase of meiosis. In *Lilium* pollen mother cells Herbert Stern and his colleagues Yasuo Hotta and Stephen Howell (1971) have not only obtained evidence which suggests that at least part of the DNA synthesised at meiotic prophase is made

by a repair-type mechanism, and so could be the single strands of DNA supposedly synthesised during recombination, but they have also found that during meiotic prophase, and at no other time, three specific enzymes are present; these are an endonuclease, a kinase and a ligase, just the enzymes required if recombination proceeds by a hybrid DNA mechanism.

In eucaryotic organisms the existence of hybrid DNA has been deduced largely from the genetic analysis of tetrads; in bacteria and viruses this type of analysis is not possible, but it has been possible to deduce the existence of hybrid DNA and of error correction mechanisms by other methods. We cannot

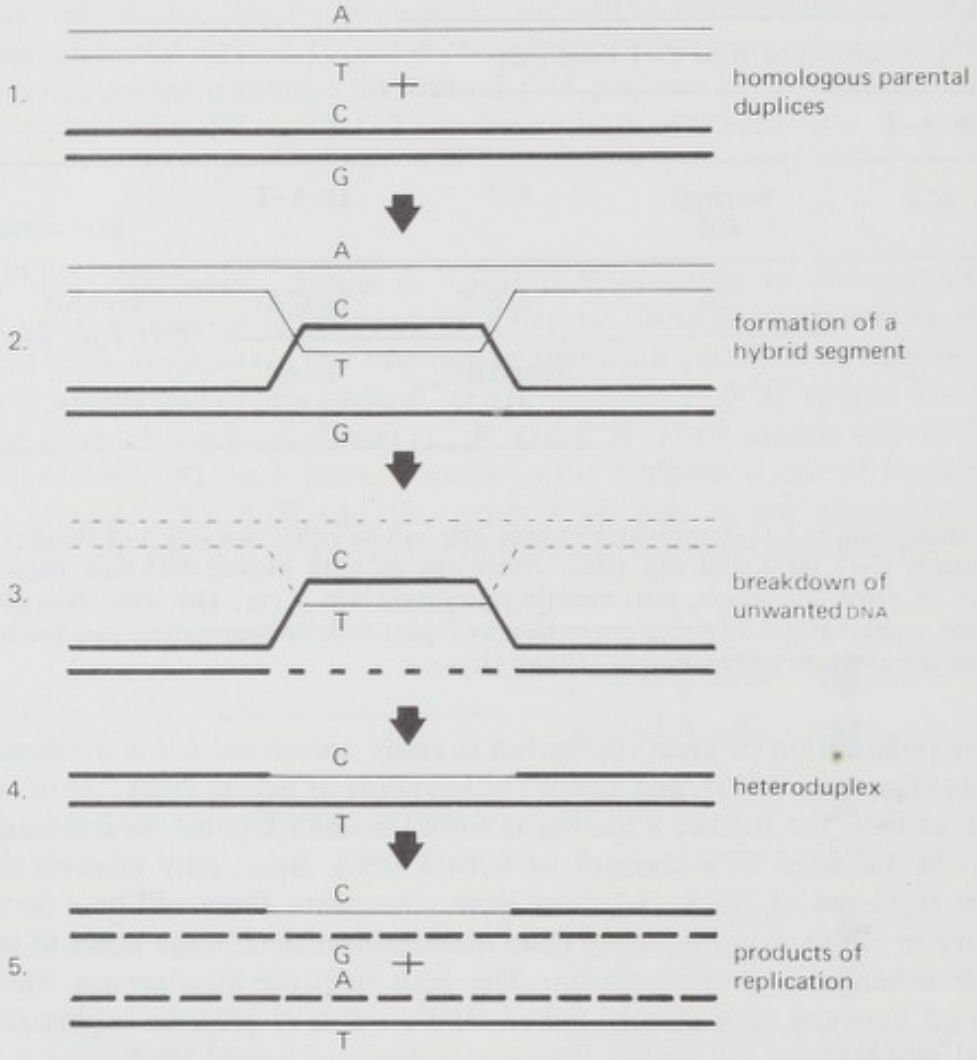


Figure 16.10 A model for the formation of heteroduplex DNA.

(1) Two homologous molecules of DNA, shown as differing by one base pair.

(2) Both parental duplices partly dissociate and single strands of opposite polarity anneal by complementary base pairing.

(3) The remaining single-stranded segments and the fragments of double-stranded DNA are enzymatically digested.

(4) DNA ligase seals the single strand gaps to form an intact but partially hybrid molecule of DNA–a heteroduplex molecule.

(5) Replication produces two daughter molecules, one identical to each of the two parental molecules.

discuss these methods at any length, but the following four points are of particular interest.

(1) During the replication of phage T4 it appears that the phage DNA molecules are broken and rejoined so giving rise to recombinant genomes with up to twenty exchanges per genome. The significant points of this process are, firstly, that the joining up of these fragments is an enzyme-dependent process, and, secondly, that recombination may sometimes involve the formation of lengths of hybrid DNA up to 1000 base pairs long; these hybrid segments are apparently due to the insertion of single-stranded DNA into the DNA duplex, any sites of heterozygosity segregating the next time the recombinant molecule replicates.

(2) In bacterial transformation the transforming DNA can only penetrate the recipient cell if it is double stranded, but it seems that only a short *single-stranded segment* of this DNA is incorporated into the duplex DNA of the recipient cell—this again produces a region of hybrid DNA, and when the bacterium next divides it produces one daughter cell with the recipient and one with the donor markers.

One possible way by which these internal segments of hybrid DNA could arise is shown in figure 16.10.

(3) Thomas Broker and I. Robert Lehman (1971) infected *E. coli* with mutants of T4, and some twenty minutes later they found branched and sometimes H-shaped molecules of duplex DNA in the infected cells; their evidence suggested that these molecules were intermediates in the recombination process. This idea was supported by the finding that when genetically defective strains of T4 that had a decreased ability to undergo genetic recombination were

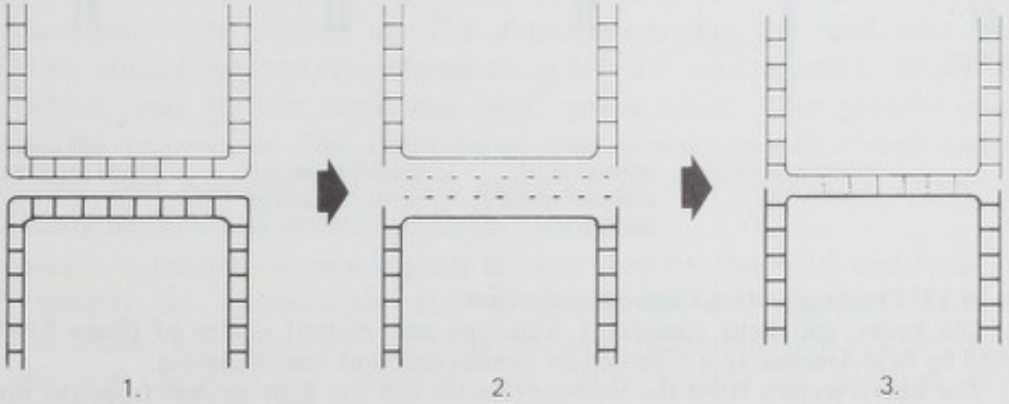


Figure 16.11 Intermediates in T4 recombination. H-shaped molecules observed by Broker and Lehman during the replication of T4 in an *E. coli* are thought to be intermediates in phage recombination. The figure shows one possible origin for these complex molecules.

(1) Two parental molecules of duplex DNA synapse along a short part of their length.

(2) Within this synapsed region strands of opposite polarity are broken down in each parental molecule.

(3) The unbroken and base complementary strands anneal, forming a short segment of heteroduplex DNA.

The cross bars represent the hydrogen bonds between the two strands of the DNA duplices.

used, a decreased frequency of these branched molecules was observed. Broker and Lehman suggested that these H-shaped molecules were complex structures made up of two parental molecules in the process of recombining, the crossbar of the H corresponding to a segment of hybrid DNA up to 2000 base pairs long (figure 16.11).

(4) Although they were not carrying out the first experiment of its type, H. Ch. Spatz and Thomas Trautner (1970) used an ingenious method to demonstrate the existence of a mechanism that corrects mispaired bases. Starting with a wild-type and a mutant strain of the *Bacillus subtilis* phage SPP1, they

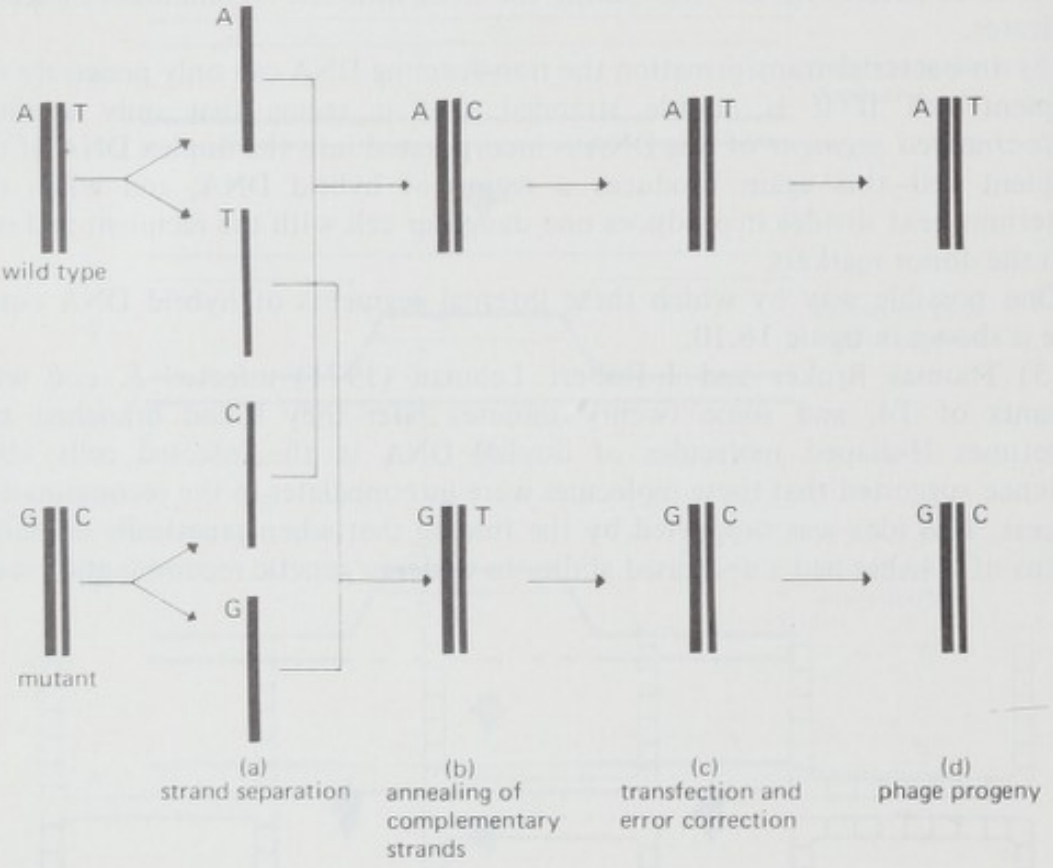


Figure 16.12 Error correction during transfection.

(a) The heavy and light strands of wild-type and mutant strains of phage SPP1 are separated by heat denaturation followed by density-gradient centrifugation.

(b) The heavy strands from the wild-type strain and the light strands from the mutant strain are annealed to make heteroduplex molecules. Reciprocal heteroduplex molecules are made by annealing the other pair of complementary strands.

(c) Each species of heteroduplex molecule is used to transfect a *Bacillus subtilis* host cell.

(d) Most of the cells transfected by one species of heteroduplex produce only wild-type phage progeny while cells infected by the other species of heteroduplex produce predominantly mutant phages. Thus, error correction seems to precede replication of the heteroduplex molecules, and, in this experiment, has been predominantly in the direction of the nucleotide sequences carried by the heavy strands.

In the diagram the mutant strain is assumed to have arisen from the wild type by an A–T→G–C transition mutation.

separated the heavy and light strands by density-gradient centrifugation (SPP1, like SP8, has a heavy purine-rich and a light pyrimidine-rich strand of DNA) and annealed the heavy strand of the wild-type strain with the base-complementary light strand of the mutant, and vice versa (figure 16.12). These reciprocal heteroduplex molecules were then used to infect *B. subtilis* and the phage particles formed in each infected bacterium were examined (this process, called *transfection*, is similar to the uptake of transforming DNA by a recipient cell). Spatz and Trautner found that, whereas most of the cells transfected by one species of heteroduplex DNA produced mainly mutant phages, the cells transfected by the other species, the reciprocal heteroduplex, produced predominantly wild-type phages; only a small proportion of the cells yielded *both* mutant and wild-type progeny phages. It is quite clear that the mispairing of bases along the heteroduplex was corrected *before* replication commenced and, furthermore, that the correction was not at random; thus in one particular experiment it was mainly in the direction of the nucleotide sequences carried along the heavy strands.

General and Site-Specific Recombination

General recombination is the term used to describe exchanges between homologous molecules of DNA, and it is this process that the polaron–hybrid DNA model seeks to explain; it occurs either at random or from very many linkage points distributed all around the chromosome. It is, as we have already seen, an enzymatic process, and some of the genes involved in the production of these enzymes have been identified in bacteria and phages. In *E. coli* there are at least nine genes involved in the control of recombination and mutations within them produce strains that are deficient in their ability to carry out general recombination. Most studied are the mutations within the *recA* gene, which completely abolish general recombination in Hfr × F^- crosses and in P1-mediated transduction, and in the *recB* and *recC* genes which have greatly reduced capacities to recombine. The function of *recA* is unknown, but *recB* and *recC* specify the two polypeptides which make up an ATP-dependent DNase with both exonucleolytic and endonucleolytic properties.

In phage λ, general recombination is promoted by the *redA* and *redB* genes which specify an exonuclease and a special protein required for recombination. λ can recombine by using the bacterial recombination system, but in a *recA E. coli* host λ *red*$^-$ mutants are quite unable to undergo general recombination.

Site-specific recombination is the name given to the particular type of recombination event which integrates and excises phage genomes into and out of the bacterial chromosome. With λ the integration of the phage genome into the *E. coli* chromosome depends on the product of the *int* gene, which specifically promotes recombination between the *att* sites on the host and phage chromosomes (chapter 9), while prophage excision also requires the *xis* gene product.

Site-specific recombination may take place by a rather different mechanism,

for whereas general recombination occurs between regions of genetic homology, site-specific recombination occurs between regions of non-homology; the P P′ and B B′ attachment sites are known to have non-homologous nucleotide sequences at least twelve nucleotides long, and the *int* and *xis* gene products presumably act by recognising specific pairs of these sequences.

The Repair of DNA

At one time it was thought that if DNA were damaged or contained an anomaly such as a mismatched base pair then either the cell would be unable to divide or it would produce mutant daughter cells (in the same way as does post-meiotic segregation). In recent years it has become clear that this is not so and that a cell is able to repair many anomalies in its DNA; this repair is sometimes only possible because the information that was in the damaged strand can be recovered from the undamaged complementary strand.

Damage by U.V.-Irradiation

Although many different agents are able to damage DNA (see chapter 10), much of our knowledge of repair systems has come from studies using mutant strains of bacteria which have lost the ability to carry out a particular type of DNA repair; these strains are unusually sensitive to u.v.-irradiation as the irreparable damage causes the death of the cells. The primary effect of the u.v.-irradiation is a photolytic action causing adjacent pyrimidine rings, particularly thymines, to coalesce in pairs; these pyrimidine dimers (figure 16.13) weaken the bonds between the two strands of the double helix and distort its architecture and severely inhibit both the replication and the transcription of the irradiated DNA. A DNA strand containing a pyrimidine dimer cannot properly act as a template for DNA replication and a single-strand gap is left in the newly synthesised strand opposite and on both sides of the dimer. A dose of 100 nJ/mm^2 is sufficient to produce about six pyrimidine dimers per 10^7 nucleotides, or about six-seven dimers per *E. coli* genome.

Photoreactivation

One of the most striking examples of a repair mechanism is photoreactivation, first observed by Albert Kelner in 1949. He was surprised to find that when actinomycetes were given a large dose of u.v.-irradiation the number of survivors could be dramatically increased by exposing the irradiated bacteria to intense visible light. Much later it was shown that photoreactivable strains produce an enzyme which, when activated by light, is able to uncouple the pyrimidine dimers, so reversing the effect of the u.v.-irradiation. Although a wide range of organisms are photoreactivable, some are not. In a simple but elegant experiment, Claud Rupert was able to show that although *Haemophilus* is not normally photoreactivable it can be reactivated by treatment with an extract

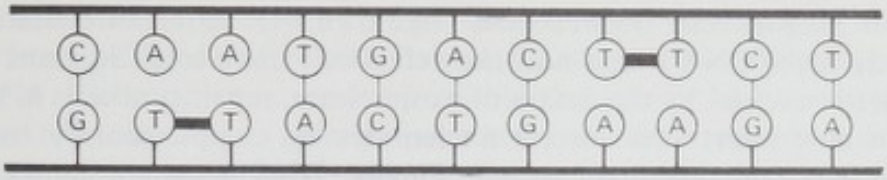


Figure 16.13 Pyrimidine dimers. The primary effect of u.v.-irradiation on DNA is to cause adjacent pyrimidines to coalesce. The diagram shows two thymine dimers.

from a photoreactivable strain of *E. coli*. After exposing streptomycin-resistant *Haemophilus* to uv-irradiation, he extracted the DNA and found that it could transform streptomycin-sensitive cells to streptomycin resistance with only about one-tenth the efficiency of DNA extracted from unirradiated bacteria. This transforming activity could not be increased by exposing the irradiated bacteria to visible light before extracting the DNA—thus *Haemophilus* is unable to photoreactivate damaged DNA. However, when the irradiated DNA was treated with an extract from *E. coli* in the presence of visible light, the transforming activity increased to 10–50 per cent of the control activity—quite clearly the *E. coli* cells contain a non-species specific enzyme able to photoreactivate DNA. Photoreactivation systems have been found in many organisms from bacteria to marsupials but not, apparently, in placental mammals. Note that photoreactivation involves only a single strand of DNA and, unlike excision repair, does not require the presence of an intact complementary strand.

Excision Repair

Excision, or dark, repair has been described in bacteria and some phages and probably exists in most other organisms; in this process light plays no part and even in the dark damaged DNA is repaired and recovers its ability to replicate. The normal wild-type strain of *E. coli* cannot only repair u.v.-induced damage to its own DNA, but it can also repair the DNA of certain irradiated phages, such as T1 and T3, which are unable to repair their own DNA (other phages, such as T4, are not dependent on the host repair system and control the repair of their own DNA); the wild-type *E. coli* is said to cause *host cell reactivation* of the irradiated phage and its phenotype is designated Hcr^+. There are a number of Hcr^- mutant strains which are unable to reactivate irradiated phage DNA and which in addition are much more radiation sensitive because they are unable to repair their own DNA. It is thought that these Hcr^- strains lack one of the specific enzymes involved in the repair process; in *E. coli* at least three widely spaced loci, *uvrA*, *uvrB* and *uvrC* (*uvr* for u.v.-repair), are involved in dimer excision.

Following u.v.-irradiation of an Hcr^+ strain the excised dimers are found associated with small fragments of DNA, and it seems that the complete excision-repair process involves a single-stranded segment about seven nucleotides long. The enzymatic steps involved in excision repair are well established from *in vitro* experiments using purified enzymes isolated from

E. coli or *Micrococcus lysodeikticus* (figure 16.14). After an endonuclease has cut the damaged DNA strand near the defective bases a small segment containing the dimer is excised by the action of exonuclease; repair synthesis in the 5′ to 3′ direction now inserts the correct nucleotides by complementary bases pairing with the bases on the intact strand. Finally, DNA ligase covalently bonds the new segment of DNA to the old, restoring the double helix.

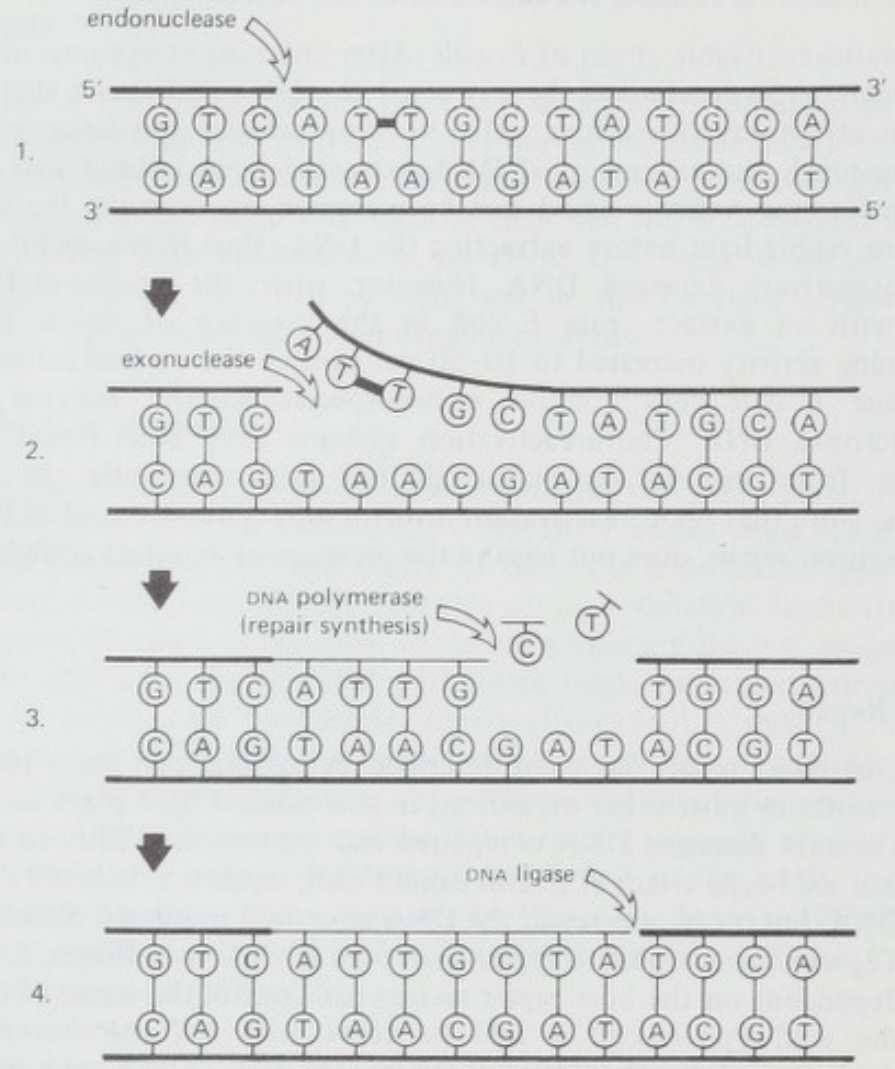


Figure 16.14 Excision repair.
(1) The damaged polynucleotide strand is nicked by an endonuclease adjacent to the defective bases (shown as a thymine dimer).
(2) The gap is widened by exonuclease.
(3) Repair synthesis inserts the correct nucleotides by complementary base pairing. The synthesis is in the 5′ to 3′ direction.
(4) DNA ligase seals the gaps, restoring the double helix.

A particularly interesting example of what appears to be a defect in an excision-repair mechanism is found in the rare inherited condition in man known as xeroderma pigmentosum, resulting from homozygosity for a recessive mutation at any one of several loci. Affected individuals are extremely sensitive

to sunlight and have a greatly increased incidence of skin cancer, and skin fibroblasts from affected individuals have a defect in their ability to repair damaged DNA. These skin cancers are probably the result of somatic mutations (see chapter 17) and their greatly increased incidence among individuals suffering from xeroderma pigmentosum shows that the DNA repair systems play a very important part in protecting us against the action of mutagenic and carcinogenic substances present in our environment—it appears that in man the efficient repair of DNA is necessary for normal cell growth and reproduction.

Note that excision repair is dependent on the presence of an undamaged complementary strand of DNA.

Recombination Repair

The observant reader will have noted that both recombination and repair require an endonuclease to nick *one* strand of the DNA duplex, that both involve the exonucleolytic breakdown of short segments of DNA, that both require the synthesis of short segments of single-stranded DNA to replace any gaps in the duplex, and that both require ligase to covalently bond the new and the old strands together. This led to the prediction that strains might be found which lacked a common protein and so would be more radiation sensitive on the one hand and unable to carry out genetic recombination on the other. Strains of *E. coli* with just these properties were first isolated by Alvin Clark and A. D. Margulies in 1965; not only were these Rec^- mutants either unable (*recA*) or had a reduced capacity (*recB* and *recC*) to form genetic recombinants, but they were very sensitive to u.v.-irradiation. They were unable to carry out another type of repair process, called recombination repair. Excision repair and recombination repair can be distinguished because excision repair takes place in the absence of replication while recombination repair only takes place during or after replication. If a culture of an Hcr^+ Rec^- strain of *E. coli* is irradiated and stored in buffer before plating, the survival is increased compared to a culture that is plated immediately after irradiation as some of the damaged DNA has been repaired; on the other hand, the storage of a Hcr^- Rec^+ culture has no effect on survival as repair only occurs during replication. The mechanism of recombination repair is not understood, but it probably involves genetic recombination between two differently damaged molecules of DNA so as to restore at least one of the daughter strands—it is the only repair system that can repair DNA damaged in *both* strands.

Paul Howard-Flanders has shown that the double mutant *uvrA recA* (that is, Hcr^- Rec^-), unable to carry out either excision or recombination repair, is very much more sensitive to u.v. (x 2500) than the wild-type uvr^+ rec^+. With the wild-type strain there is 37 per cent survival after a u.v. dose of 50 $\mu J/mm^2$ and an average of 3200 induced dimers per bacterial genome, but with the double mutant *uvrA recA* there is 37 per cent survival after a dose of only 20 nJ/mm^2 and an average of only 1.3 dimers per genome. Because of the inability of this strain to carry out either excision or recombination repair, the induction of a very small number of dimers per genome is lethal. The single mutants *uvrA* rec^+ and uvr^+ *recA* have intermediate sensitivities.

DNA Repair and Mutation

The ability or inability of the cell to carry out DNA repair can also have a significant effect on the frequency of mutations induced by u.v.-irradiation (and also on similar mutations induced by other mutagens). Evelyn Witkin compared u.v.-induced mutagenesis in Hcr^+ and Hcr^- strains of *E. coli*. Both strains were given a dose of 2 $\mu J/mm^2$, sufficient to induce about 120 pyrimidine dimers per genome. In Hcr^- cells, all or most of these dimers remained uncorrected and about 200 mutations to streptomycin resistance were found among every 10^7 bacterial survivors, but in Hcr^+ cells the corresponding mutation frequency was 0.4 mutations per 10^7 survivors. This shows that not only are most u.v.-induced mutations due to the continued presence of the pyrimidine dimers but also that the excision-repair process in Hcr^+ cells must itself be very accurate; Witkin calculated that the excision-repair mechanism makes less than one error for every 10^6 dimers excised.

Witkin also found that u.v.-irradiation cannot induce mutations in *recA* strains while *recB* and *recC* strains produce fewer mutations than do wild-type strains. All these results are consistent with the notion that u.v.-induced mutations are induced *after* replication and during the recombinational repair of any pyrimidine dimers that have failed to be excised by the excision-repair process; this suggests that recombination repair makes more mistakes than does excision repair.

Summary of Key Points

(1) The results of tetrad analysis in Ascomycetes show that while meiosis is normally a reciprocal process, a small but predictable proportion of the tetrads contain non-reciprocal products that have arisen by the process of gene conversion.

(2) Gene conversion has also been detected in *Drosophila*.

(3) The polaron-hybrid DNA model for recombination accounts for both reciprocal and non-reciprocal recombination in higher organisms by the breakage and unwinding of complementary strands of paternal and maternal origin followed by their annealing to form short segments of hybrid DNA. Markers falling within this segment may show non-reciprocal crossing-over while markers spanning the segment will show normal reciprocal recombination.

(4) Sites of heterozygosity within this hybrid DNA may be repaired by an error-correcting mechanism or they may persist and give rise to post-meiotic segregation.

(5) In microbial systems there is considerable evidence for the occurrence of short segments of hybrid DNA that are intermediates in the recombination process, and for the occurrence of an error-correcting mechanism.

(6) Damaged DNA can be repaired by one of three repair mechanisms: (i) photoreactivation, which cleaves apart pyrimidine dimers and does not require an intact complementary strand of DNA; (ii) excision repair, which removes

dimers from a damaged molecule and replaces them with the correct complementary nucleotides—it requires an undamaged complementary strand; and (iii) recombination repair, which occurs during and after replication and can repair DNA damaged in both strands.

(7) Bacterial mutants deficient in recombinational repair are not susceptible to mutation by u.v.-irradiation.

17 Mutation and Gene Expression

Why is this thus? What is the reason of this thusness?
Artemus Ward (1834–1867)

Gene Suppression

In previous chapters it has been assumed that reversion usually occurs by back-mutation at the mutant site which exactly restores the nucleotide sequence of the wild-type gene. This is certainly true for many mutants, but there are numerous examples in micro-organisms and higher organisms where the harmful effect of a mutation is overcome by a second, or *suppressor,* mutation, which occurs at a different site; these suppressed mutants are double mutants, containing both the original mutation and the suppressor mutation, so that the original mutant phenotype can be recovered by making the appropriate genetic crosses. Suppression is of quite common occurrence in microbial systems, so that special care is necessary if mutation data are to be meaningfully interpreted; for example, if we are measuring the rate of mutation from auxotrophy to prototrophy in bacteria, the data may be of little value unless reversions due to back-mutation are distinguished from those due to suppressor mutations.

Although there are special cases where a suppressor mutation provides an alternative metabolic pathway, so by-passing the block due to the original mutation, most suppressors act by enabling the *mutant* gene to produce an *active* protein; a detailed study of suppression has yielded valuable information on the translational process as well as revealing the mechanism of suppression. Suppression is called intragenic when both mutations are within the same gene and intergenic when they are in different genes.

Intragenic Suppression

Both frameshift and missense mutations can be suppressed by intragenic suppressors. Frameshift mutations involve the addition or deletion of one or more base pairs so that the code is read in phase up to the codon containing the mutation and out of phase thereafter. If the mutation is due to a base-pair addition (+) a deletion mutation (–) in a nearby codon can often result in the production of an active protein, as the entire gene is now read in phase; provided that the codons between and including the mutant sites do not include a nonsense triplet they will all be read as missense, but this need not significantly affect the activity of the protein. The suppression of frameshift mutations in phage T4 was fully described in chapter 12.

Intragenic suppression of missense occurs when two missense mutations within the same gene result in the production of an *active* protein. A good

Table 17.1 The intragenic suppression of missense in the *trpA* gene of *E. coli*

Position	Tryptophan synthetase A protein 174	175	176	210	211	212	Codon change
wild type	Tyr	Leu	Leu	Gly	Phe	Gly	
trpA46	Tyr	Leu	Leu	**Glu**	Phe	Gly	GGA or GAA
trpA446	**Cys**	Leu	Leu	Gly	Phe	Gly	$UA\frac{U}{C}$ or $UG\frac{U}{C}$
trpA446 + *trpA46*	**Cys**	Leu	Leu	**Glu**	Phe	Gly	

The *trpA446* and *trpA46* strains are both auxotrophic because of different missense mutations. When both these mutations are combined into the same gene a functional protein is produced. The substituted amino acids are printed in bold type.

example is in the *E. coli* tryptophan synthetase A protein studied by Charles Yanofsky (table 17.1). By itself neither *trpA446* nor *trpA46* produces an active A protein because the resulting amino-acid substitution has altered the three-dimensional configuration of the protein and inactivated it. However, when both these mutations are combined together in the same gene, the two substituted amino acids act together in such a way that the original configuration and biological activity of the protein is restored. This phenomenon is not to be confused with intragenic complementation (page 192)where the complementing genes are on *different* chromosomes and the interaction is between two differently altered protein molecules.

Intergenic Suppression

Intergenic suppression is sometimes called informational suppression because its explanation lies in the way that genetic information is transferred from gene to protein. The best-known ways by which it occurs are by alterations either to the translational mechanism, so affecting the specificity of translation, or to the ribosomal components, and consequently affecting the accuracy of translation (see table 17.2).

The best-understood intergenic suppressors are the suppressors of nonsense found in *E. coli*. Each suppressor enables a nonsense codon to be read just as if it were a sense codon for a specific amino acid so permitting the growing polypeptide to be extended beyond the nonsense (chain termination) triplet to the end of the gene. Each nonsense mutation can suppress nonsense mutations not only in many different *E. coli* genes but also in the genes of infecting phages; this is not unexpected as a mechanism which causes the misreading of the mRNA code can just as easily misread a nonsense codon in one gene as in

Table 17.2 The genetic basis of suppression

Mutation	Effect	Intergenic Suppression	Intragenic Suppression
Missense	Causes an amino-acid substitution that inactivates the polypeptide	Mutation in a particular tRNA changes the anticodon so that the tRNA now inserts the original amino acid at the mutant codon	A second missense mutation in a different codon causes a second amino acid substitution which complements the first mutation so that the polypeptide now can assume an active 3D configuration
Frameshift	The code is read out of phase from the point of an addition or deletion mutation	Mutation in a tRNA gene modifies a tRNA so that it can recognise a four- or five-letter codon and restore the phase of the code	Deletion (or addition) of a base pair adjacent to the first mutation restores the reading phase of the code
Nonsense	Premature polypeptide termination due to the generation of a chain-termination triplet	Mutation in a tRNA gene changes the tRNA anticodon so that it can recognise a nonsense codon	Not known, except as a result of a further base-pair substitution within the same codon, so changing nonsense back to sense

another, and the only occasion when suppression will be ineffective is when the inserted amino acid disrupts the three-dimensional configuration of the protein and inactivates it. There are three types of nonsense suppressor and these suppress amber mutants only, UGA mutants only or both amber and ochre mutations.

The mechanism of this suppression is quite straightforward. The suppressor mutations occur in the structural genes for tRNA and alter the anticodon so that the mutant tRNA, instead of recognising its own codon on the messenger, now recognises a nonsense codon. For example, the *su3*$^+$ suppressor (active suppressors are conventionally designated *su*$^+$) is a mutation within a tyrosine tRNA gene that changes the anticodon from GUA, which recognises the tyrosine codon, to CUA, which recognises the amber codon UAG. We may now ask how the cell continues to insert tyrosine at the UAU and UAC codons, and why this change is not lethal to the cell. Again the answer is simple. Most species of tRNA are coded by more than one gene; thus, in addition to a gene specifying a major species of tyrosine tRNA, two further genes specify a minor tyrosine tRNA (page 247) and so a change within one of the minor tRNA genes should not significantly influence the reading of the tyrosine codons. On the other hand, there is only *one* gene which encodes tryptophan tRNA and a change in its anticodon is lethal to the cell as virtually all proteins contain tryptophan. Suppressor mutations (*su7*) do occur within this gene, but they can only be

Table 17.3 Suppressors of nonsense in *E. coli*

Suppressor	Map position (min)	Amino acid carried by suppressing tRNA	Actual* or inferred anticodon change	Codons suppressed
su1	38	serine	CGA* → CUA	UAG amber
su2	15	glutamic acid	CUC → CUA	UAG amber
su3	24	tyrosine	GUA* → CUA	UAG amber
su7	75	glutamic acid	CCA → CUA	UAG amber
su4	24	tyrosine	GUA → UUA	UAA ochre UAG amber
su5	15	lysine ?	UUU → UUA?	UAA ochre UAG amber

The *su7*$^+$ mutation is in the tryptophan tRNA gene and alters not only the tRNA anticodon but also the amino acid it carries. The other suppressors only alter the tRNA anticodons.

studied in F′ partial diploids; note that the *su7*$^+$ mutation (table 17.3) also changes the amino acid carried by the tRNA.

The amber suppressors are relatively efficient and 30–60 per cent of the polypeptides initiated by the mutant gene are completed. The ochre suppressors, however, have only a low efficiency of suppression (1–15 per cent chain completion) and furthermore they will suppress amber mutations with low efficiency. The explanation of this pleiotropic effect is that the UAA ochre codon is presumably recognised by the anticodon UUA and, according to the wobble hypothesis (page 246), this can pair with either UAA (ochre) or UAG (amber).

Several intergenic suppressors of missense are known in the *E. coli* tryptophan synthetase A protein gene and are the result of mutation in one of the several glycine tRNA genes. For example, the *trpA36* auxotroph is the result of a glycine → arginine (GGA → AGA) substitution at a position within the A protein and it can be suppressed by a mutation (*su36*$^+$) which changes the anticodon of glycine I tRNA so that it now recognises the AGA codon and inserts the wild-type amino acid, glycine. This suppression is only weakly effective as 95 per cent of the complete A protein molecules are inactive and still have arginine at the position of the substitution.

In 1968 Shahla Ryce and John Atkins discovered that frameshift mutations in *Salmonella typhimurium* could also be reverted by external suppressors which alter a tRNA in such a way that it can restore the correct phase for translation. The six known suppressors are all specific and do not suppress either missense or nonsense mutations and they arise by mutation in genes responsible for the production of tRNA. Although the sequence changes in the tRNAs are unknown these suppressors can be induced by acridine treatment and so may have additions or deletions in their nucleotide sequences enabling them to translate as sense a codon containing four or five nucleotides.

Alterations in Ribosomal Structure cause Misreading of the Code

In order for the code to be read accurately, the ribosome must be intact so that any distortions, whether caused by mutations in the genes coding the structural components of the ribosomes or by interaction between the ribosomes and an external factor, may cause misreading of the code. In *E. coli* there are several mutations which affect one or other of the 30S ribosomal subunits; their effect is to alter the ribosomal structure so that the mRNA-ribosome complex can sometimes select the wrong tRNA molecule and so insert the wrong amino acid into protein. This misreading of the code can sometimes cause weak suppression—for example, the *ram* (*r*ibosomal *am*biguity) mutation occasionally allows nonsense codons to be read as sense even though there are no suppressors present. Streptomycin can also cause misreading of the code by binding to the ribosomes and disturbing the mRNA-ribosome complex; this is called phenotypic suppression. If streptomycin is added to sensitive *E. coli* cells (*str*^{s}) very many misreadings occur but with streptomycin-resistant (*str*^{r}) cells misreading is much less frequent, although frequent enough to cause the weak

suppression of some missense mutations. It turns out that these mutations all occur at a single *str* locus and alter the 30S core protein in such a way that interaction with streptomycin is minimised; thus the *str* gene is probably the structural gene for the 30S core protein.

Mutation and Cancer

Many chemical mutagens are, like ionising radiations, also carcinogenic and, indeed, some compounds were first tested and recognised as chemical mutagens because they were known to cause tumours in experimental animals. What, therefore, is the connection between mutation and cancer?

The term 'cancer' includes many different diseases as cancers can arise in almost any differentiated cell, whether dividing or not, but all have one common property; the cancer cells divide more rapidly than the normal cells and so eventually grow into a tumour. Cancer is due primarily to a change in the *rate* of cell division, and it appears that the complex control mechanism which tells the cells when or when not to divide has broken down. Some tumours only grow very slowly, but others grow rapidly and so if not removed by surgery or treated by radiation or chemicals will kill the organism. The cancer cells may remain in a localised tumour, often removable by surgery, but other cancer cells become invasive and spread rapidly through the organism causing tumours in many different tissues; unless these invasive tumours can be detected very soon after they arise they cannot be treated or removed by surgery and will nearly always lead to the death of an organism.

When a cancer cell divides it produces two identical daughter cells which, like the parent cell, can continue to divide out of hand; these changes also persist in tissue culture or when cancer cells are taken from an individual and injected into a genetically similar individual. Thus the changes that produce unrestricted growth appear to be heritable. Several theories have been suggested to account for this change, but perhaps the most intellectually satisfying is that many cancers are due to somatic mutations occurring, possibly, in the genes controlling the rate of cell division; it is not clear how many somatic mutations would be necessary to cause a cancer but the fact that the incidence of cancer increases with age suggests that cancer might be the consequence of several harmful mutations accumulating in the same somatic cell. If this theory is correct then all compounds that induce cancer (that is carcinogens) should also be able to induce mutations. This possibility has been investigated by Bruce Ames (1973) who has used specially constructed tester strains of *Salmonella typhimurium* to test the mutagenicity of a wide range of known carcinogens. These tester strains contained three mutations. Firstly, a mutation causing a defect in the cell envelope, so ensuring that the carcinogen has the maximum chance of getting into the bacterial cell; secondly, a mutation in the DNA excision-repair system, so minimising the repair of any genetic damage caused by the carcinogens; and thirdly, a mutation within the histidine gene cluster; the effectiveness of a carcinogen as a mutagen was assessed by scoring the number of histidine-independent reversions induced on a minimal medium plate.

Ames tested each carcinogen against four tester strains—TA1535 containing a base-substitution mutation, TA1536 containing a frameshift mutation in a nucleotide sequence that is particularly susceptible to the frameshift mutagen ICR (page 175) and its derivatives, TA1537 containing a frameshift mutation within a repetitive sequence of G–C base pairs, and TA1538, a frameshift mutation within the sequence.

GCGCGCGC
CGCGCGCG

Many carcinogens are known to be activated by hydroxylase systems present in mammalian liver cells but absent in bacteria—thus the actual carcinogen is often the metabolic product of an ingested secondary carcinogen. To overcome this difficulty Ames added an extract from liver cells to each petri dish containing the tester bacteria and the carcinogen. The results of some of these experiments (table 17.4) show several features of interest. Firstly, only 2-aminoanthracene and 2-naphthylamine were able to induce base-substitution mutations in TA1535. Secondly, only 2-aminoanthracene induced the reversion

Table 17.4 Carcinogens are mutagens

Carcinogen	Experiment	His^+ reversions per plate containing about 10^9 bacteria of the tester strain			
		TA1535	TA1536	TA1537	TA1538
2-aminoanthracene	liver extract only	16	3	12	46
	carcinogen only	21	0	17	27
	carcinogen + liver extract	*318*	*11*	180	*11200*
2-naphthylamine	liver extract only	16	2	18	21
	carcinogen only	20	0	8	15
	carcinogen + liver extract	*330*	2	34	*85*
benzopyrene	liver extract only	48	1	28	44
	carcinogen only	77	1	16	34
	carcinogen + liver extract	46	4	*148*	*505*
7,12-dimethyl-benzanthracene	liver extract only	29	1	11	36
	carcinogen only	35	0	15	20
	carcinogen + liver extract	25	1	*225*	*88*

Bruce Ames (1973) has tested many known carcinogens to see whether they are mutagenic in bacteria. The table shows some of his results using four tester strains of *Salmonella typhimurium* containing different *his* mutations. TA1535 is a base-substitution mutant, the other testers contain frameshift mutations within different nucleotide sequences. Significant increases in the frequency of reversion are shown in italics.

of TA1536, and then only very weakly. Thirdly, the remaining carcinogens either reverted TA1537 strongly and TA1538 weakly, or vice versa. Not only were all the carcinogens tested also mutagenic, but it also appears that different mutagens have different affinities for particular nucleotide sequences. It is conspicuous that the most potent carcinogens strongly induce frameshift mutations; if the somatic-mutation theory is correct, this is not unexpected, as not only would frameshift mutations be incapable of repair by the highly efficient excision-repair systems, but also mutagens which cause base-substitution mutations (such as the alkylating agents) are more likely to be inactivated before they reach the target cell.

This technique is of considerable practical importance as it is an ideal method for the rapid screening of suspected carcinogens that may act by inducing point mutations. Not only is it a very sensitive test, but it is very cheap, and the results are available within two days, instead of the weeks that are involved when a higher-organism test system is used.

Viruses and Cancer

Although Ames's experiments show that there is a more than reasonable probability that somatic mutation is the cause of at least some cancers it must be noted that tumours can also be virus-induced. For example, the RNA-containing Rous sarcoma virus (RSV) induces tumours in the connective tissue of chickens, while the DNA-containing polyoma and SV40 viruses can cause a variety of tumours in mice and monkeys respectively. The relationship between the host cell and a tumour virus is rather complex, but it seems that the viral genome (or with RNA viruses a DNA copy of the genome) is added to and becomes established in the host cell, causes change in the functioning of the cell and so *transforms* it into a cancer cell. This process is not dissimilar to the lysogenisation of a bacterial cell by a phage, and it could be regarded as a rather bizarre form of somatic mutation.

Because virus-like particles have been isolated from so many different tumour cells, it has been suggested that chemical carcinogens could act by activating latent carcinogenic viruses already present in the cells, but there is no evidence to support this idea.

Although tumour-inducing viruses are known in several organisms, there is still no *positive* evidence that human tumour viruses exist; nevertheless, there is now an increasing body of circumstantial evidence to suggest that they do. The most direct evidence has been obtained by Sol Spiegelman and his associates (1973) who, in their most recent experiments, studied two sets of identical twins where one member of each pair had myeloytic leukaemia while the other twin was normal. They found that both the leukaemic individuals had nucleotide sequences in their leucocytes that were absent from the leucocytes of the normal individuals and, furthermore, that these additional nucleotide sequences were partly homologous with the RNA of the Rauscher leukaemia virus, which causes cancers in mice. Since the sequence differences existed between identical twins the additional nucleotide sequences found in the leukaemic individuals must

have been added *after* the zygote was formed. These result make it most unlikely that these cancers are due to the activation of a latent virus, as then both the normal and leukaemic individuals should have had virus-like nucleotide sequences; the alternative is that a virus entered the cells of the leukaemic individuals by simple infection, and there induced cancer.

Although the evidence is hardening that some cancers are virus induced, we must always remember that the presence of a virus in a cancer cell does not necessarily mean that the virus was the cause of the cancer—one could also argue that cancer cells are more susceptible to virus infection.

Position Effect Phenomena

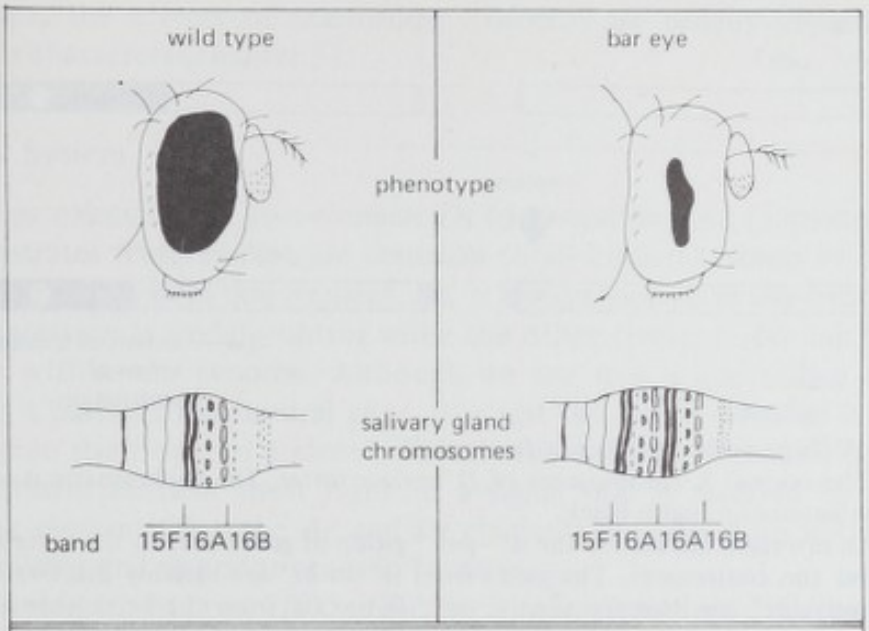
Earlier in this chapter we saw that the activity of a gene can be affected by a mutation in another gene elsewhere in the genome; whether or not a gene is active can also depend on the *position* of that gene in the genome relative to the other genes. In microbial systems it is self-evident that in order to be regulated a structural gene must be located next to its operator and promoter genes, but in eucaryotic systems there is no evidence that homologous regulatory genes exist. Nevertheless, eucaryotic genes are clearly subject to genetic regulation (chapter 18) and a study of position effects can yield useful information on the mechanism of regulation. Here we shall consider well documented examples of position effects in *Drosophila* and maize.

Position Effects in *Drosophila*

Position effects are observed when changing the position of the genes relative to each other on the chromosomes results in a change of phenotype; sometimes these changes are stable but other times they are unstable and, usually, expressed as a variegation (V-type position effects).

A simple example of a stable position effect is seen in the Bar-eye mutants of *Drosophila*. The dominant Bar-eye phenotype is the result of a small duplication in the X chromosome, and affected flies have slit-like eyes caused by a reduction in the number of eye facets (ommatidia). X chromosomes also exist that carry a triplication of this region, so making it possible to observe the effects on the phenotype of two, three or four Bar regions in different arrangements (figure 17.1). Alfred Sturtevant was the first to note that the more Bar regions that are present, the more extreme the phenotype, showing that Bar is not completely dominant, and also that, although *BB*/*BB* and *B*/*BBB* females both have the Bar region represented four times, they have different phenotypes: there is a position effect.

More interesting are the V-type position effects which may result when a gene located in euchromatin is moved by a chromosomal rearrangement to a new position adjacent to heterochromatin. The white-eye (*w*) locus is located near the end of the long arm of the X chromosome and w^+ controls the production of red-eye pigment– thus w^+/w^+ and w^+/w flies have normal red eyes. When w^+



genotype of female	$\frac{B}{B}$	$\frac{BB}{B}$	$\frac{BB}{BB}$	$\frac{BBB}{B}$	$\frac{BBB}{BB}$
average number of facets per eye	779	358	68	45	36

Figure 17.1 Bar eye in *Drosophila melanogaster*. Flies with the bar-eye phenotype (right) have very few eye facets and their eyes are reduced to narrow slits. This phenotype is the result of a small duplication involving band 16A of the X chromosome. Females homozygous for the bar duplication (*BB/BB*) have more facets per eye than females with one normal X chromosome and one containing three bar regions (*BBB/B*). These two genotypes show a position effect. The duplicated and triplicated bar regions are believed to have arisen by unequal crossing-over.

is relocated by an inversion so that it is near the heterochromatic centromere region, its pigment producing capacity is impaired, and is impaired to a variable extent even between cells within the same eye; thus a *rearranged* w^+/w heterozygote has some eye cells that have the full amount of red pigment, some that have no pigment and others that have intermediate amounts, so producing a mottled or *variegated* eye. These changes are unlikely to be due to recurrent somatic mutation, because not only is the activity of w^+ restored if it is moved back to near its original position, but there is also a *spreading effect* as other genes closely linked to w^+ show simultaneous position effects—the nearer they are to the heterochromatin, the greater the effect (figure 17.2).

The heterochromatic regions of chromosomes differ from euchromatin in several ways. Firstly, the DNA is packaged differently and condenses differently at cell division; secondly, the heterochromatic regions appear to contain very few genes of major effect; thirdly, at a functional level the heterochromatin is probably relatively inactive as a DNA template for transcription. When w^+ is transposed to near heterochromatin it sometimes takes on the appearance of heterochromatin and a possible explanation of these position effects is that the normal heterochromatic regions affect the way the DNA is coiled in the

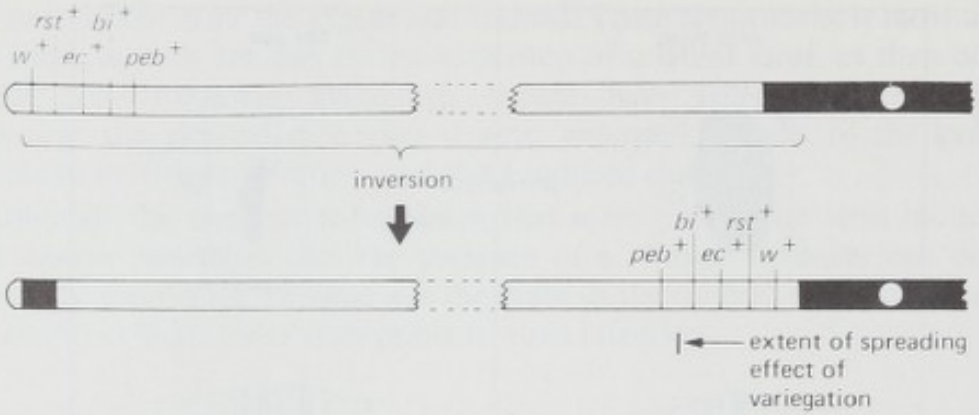


Figure 17.2 V-Type position effect in *Drosophila.*

Above: The normal X chromosome of *D. melanogaster.* The euchromatin is represented by white, the heterochromatin black.

Below: An inversion has moved the w^+–*peb*$^+$ group of genes near to the heterochromatic region around the centromere. The genes from w^+ to bi^+ are variably inactivated, w^+ the most frequently, bi^+ the least frequently; *peb*$^+$ is too far from the heterochromatin to be inactivated.

w	white: white eyes
rst	roughest: eyes are rough and bulging
ec	echinus: eyes large and bulging, the body short and broad
bi	bifid: longitudinal wing veins fused at base
peb	pebbled: eyes rough at 28°, normal at 19°.

transposed w^+ segment so that it becomes partially heterochromatinised and less available as a template for transcription; to account for the spreading effect it is only necessary to suppose that the nearer the euchromatic gene to the heterochromatin the greater is the degree of heterochromatinisation. The implication of these experiments is that the activity of a gene may depend on the way its DNA is packaged into the chromosome.

A similar situation is found when a gene normally located near heterochromatin (for example, the light-eye gene on chromosome 2) is moved to a new location near euchromatin. These V-type position effects have been described in a wide range of organisms including maize and *Oenothera.*

Controlling Elements in Maize

In 1938 Marcus Rhoades discovered a highly unstable allele of a_1 whose activity is controlled by a quite separate genetic unit located on a different chromosome. Although it was later shown that this instability was due to the presence of controlling elements, the real discoverer of controlling elements was Barbara McClintock; for over twenty years she studied controlling element systems in one of the most intricate and painstaking genetic analyses ever carried out. Controlling elements are regulatory elements which *control* and *program* the activity of the structural genes, and they have the unusual property of being able to move around, or *transpose,* within the genome; their particular importance is that they are one of the very few instances where the genetic components of a eucaryotic regulatory system have been identified. For

convenience, the effects of controlling elements are usually observed on the endosperm characters (chapter 5).

The *Ds-Ac* System

First, let us examine the two-element *Ds* (dissociation)-*Ac* (activator) system, which illustrates features that are common to all known systems of controlling elements. In this system one element, *Ds*, is always located at the structural gene whose expression is under control while the other element, *Ac*, can be located anywhere within the genome. Although we say that a controlling element is located *at* a particular structural gene, it is not yet certain whether it is inserted into the gene itself or into a closely linked region of the chromosome. In so far as the activator element must transmit a signal that is received by a sensitive responding element (*Ds*), the *Ac* and *Ds* elements are analogous in their actions to the regulator and operator genes in bacteria.

The *Ds* element has two effects. Firstly, it may sometimes cause breaks at the point on the chromosome where it is located, but it will *only* do this as long as an *Ac* element is present somewhere in the genome. Secondly, it may, but will not necessarily, partially or completely suppress the activity of the gene where it is inserted. For example, no pigment is normally produced in the endosperm unless the genes *C* and A_1 are present and a kernel of the genotype *ccC* will normally be pigmented (figure 17.3(1)) but, if there is a *Ds* element located at *C* the activity of *C* may be totally or partially suppressed (figure 17.3(2)) and the endosperm will be either colourless or weakly coloured; *C* will remain suppressed so long as *Ds* remains there.

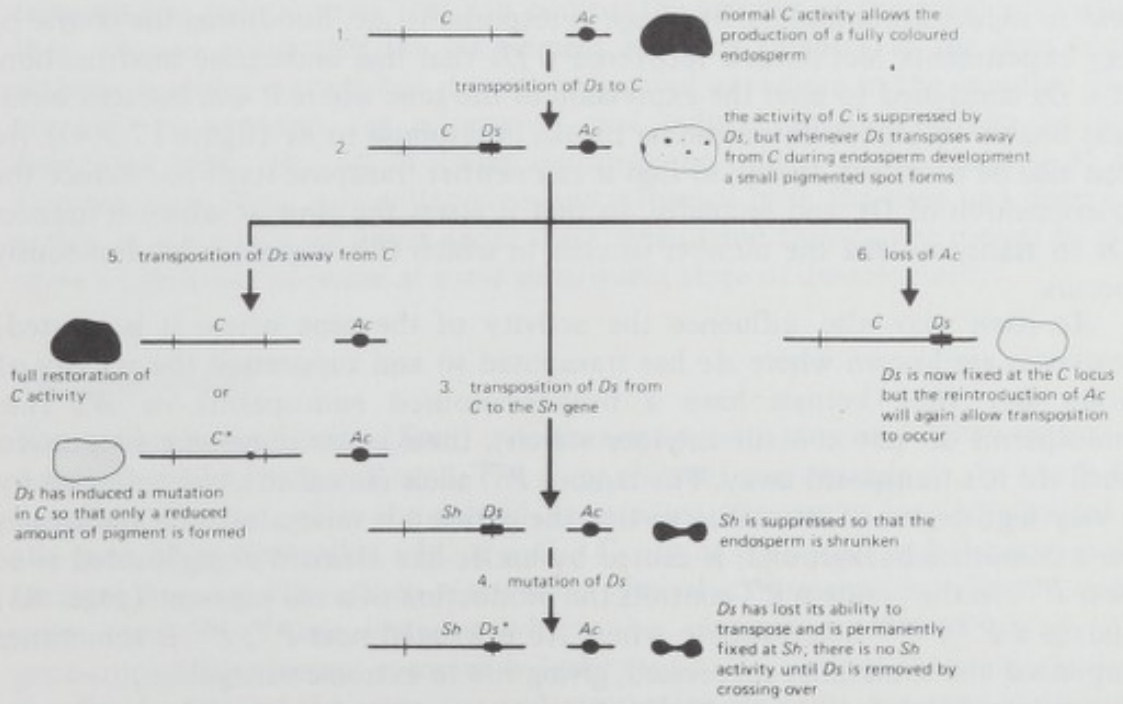


Figure 17.3 Aspects of the *Ds–Ac* controlling element system in maize.

In the presence of Ac a Ds element can either suppress the action of or cause breaks within the gene with which it is associated.

Occasionally however, so long as an *Ac* element is present, *Ds* is able to transpose its position at the *C* locus to some other location within the chromosome complement. *Ds* has been observed to transpose to the A_2, Sh_1, Bz_1 and *Wx* genes, and in each instance the transposition has been recognised because one or other of these genes has been brought under the control of the controlling element system and its activity suppressed (figure 17.3(3)). Every time that *Ds* transposes away from *C* during the development of the endosperm, the suppression of *C* is released and a spot of pigmented cells will be formed.

Ds can transpose from the C gene to some other gene within the genome; C is now released from suppression and a different gene is brought under controlling element control.

The size of the spot formed when *Ds* transposes away from *C* will depend on the number of cell divisions that takes place after the restoration of gene activity, and the later in development transposition occurs, the smaller will be the size of the spot. Sometimes the reactivated gene is qualitatively or quantitatively different from the original *C* allele, so that in the presence of *Ds* the mutation of *C* to a new allele *C** has occurred (figure 17.3(5)). Since the endosperm is a triploid tissue, it is easy to study the effect of 0, 1, 2 or 3 *Ac* elements on the expression of the genes linked to *Ds*, and it turns out that the more *Ac* elements that are present the smaller the size of the spots—thus *Ac* must also control the *time* at which transposition occurs.

Both *Ds* and *Ac* can exist in different *states* (the different states of a controlling element correspond to the different mutant forms, or alleles, of a structural gene). When *Ds* was first isolated, it was able to suppress gene activity *and* to induce chromosomal breakage in response to *Ac*, but during the course of her experiments McClintock recovered a *Ds* that had undergone modification, this *Ds* continued to alter the expression of the gene where it was inserted but it was unable to induce chromosome breaks in response to *Ac* (figure 17.3(4)). *Ac* can also be modified, firstly, so that it can neither transpose itself nor induce the transposition of *Ds*, and secondly, so that it alters the time at which it induces *Ds* to transpose *and* the number of cells in which this response simultaneously occurs.

Ac itself may also influence the activity of the gene where it is located. Instances are known where *Ac* has transposed to and suppressed the activity of either Bz_1 (bz_1 kernels have a bronze-coloured endosperm) or *Wx* (*wx* endosperms do not contain amylose starch), these genes remaining suppressed until *Ac* has transposed away. The famous P^{vv} allele (so-called), characterised by a very high degree of instability so that the pericarp is variegated with red stripes on a colourless background, is caused by an *Ac*-like element being located at or near P^{rr}. In the pericarp P^{rr} controls the production of a red pigment (page 83) and in a P^{rr}/P^{ww} heterozygote where *Ac* is located near P^{rr}, P^{rr} is sometimes expressed and sometimes suppressed, giving rise to extreme variegation.

An interesting feature of the *Ds-Ac* system is that *Ac* also undergoes *cycles of activity*; it is only when *Ac* is in the active phase that it can signal to *Ds* to

induce chromosome breaks and to transpose. One kernel examined by McClintock had the genotype

$$a_1/a_1/a_1\ Ds \qquad Wx\ Ac^*/Wx\ Ac^*\ wx$$

both *Ac* elements being inactive (*). The first-formed cells of the endosperm were uniformly colourless (since A_1 was suppressed by *Ds*) and did not contain starch (since *Wx* was suppressed by *Ac*), but during early development reactivation of one of the *Ac* elements took place. This resulted in the production of *both* coloured spots, due to the transposition of *Ds* from A_1 and starch, due to the transposition of *Ac* from *Wx*.

Finally, and of particular interest, is the phenomenon of *pre-setting*. When McClintock used a particular plant homozygous for *Ac* and *Ds* as pollen parent in crosses with over twenty plants of widely different genetic backgrounds, she observed that in nearly every kernel on each ear the *time* at which *Ds* transposed was different to what it had been in previous generations—thus the *Ac* element introduced from the pollen parent apparently underwent modification immediately after fertilisation in such a way that the time of response to *Ds* (the time of transposition) was simultaneously altered in *all* the daughter cells. Furthermore, this modified response was *not* transmitted to the following generations; it is difficult to avoid the conclusion that the *Ac* elements in the sperm nuclei (see figure 5.2) must have been *pre-set* to undergo modification immediately after fusion with the two polar nuclei contributed by the ear parent, while fusion of a sperm nucleus with a single egg nucleus to form a diploid zygote must have erased this pre-setting.

Studies of the *Ds-Ac* system thus show that these controlling elements are transposable genetic units that can control the activity of the genes with which they become associated; the *Ds* element must be adjacent to the gene whose activity is under control but *Ac* can be located anywhere in the genome. They are able to suppress the activity of, and induce mutation within the gene associated with, *Ds* and to cause chromosome breaks at the location of *Ds*. Furthermore, these controlling elements influence both the time and degree of action of the structural genes under their control and they can be pre-set so as to show a particular response at some subsequent stage of development.

The *Spm* System

The suppressor–mutator (*Spm*) two-element system reveals further aspects of controlling-element regulation. The *Spm* element corresponds to *Ac* and is the signalling element while the second, responding, element is located adjacent to the gene being controlled (A_1, A_2, C_1, C_2, *Pr*, or *Wx*). Although the responding element has not been given a name or symbol its presence is indicated in the gene symbol—in one instance where this element is located at A_1 the gene-controlling element complex is designated $a_1{}^{m-1}$. In just the same way as in the *Ds-Ac* system, the signalling and responding elements can exist in different states. In an $a_1/a_1/a_1{}^{m-1}$ endosperm that does not contain an *Spm* element, the

formation of pigment is partially or totally suppressed, the degree of suppression depending on the particular state of $a_1{}^{m-1}$ and any pigment that is formed is uniformly distributed through the aleurone (figure 17.4b). However, when *Spm* is also present ($a_1/a_1/a_1{}^{m-1}$ *Spm*), the expression of $a_1{}^{m-1}$ is completely altered; these kernels (figure 17.4a) have many small and intensely pigmented spots on a completely colourless background. Observe that *Spm* has expressed itself in two ways, first by suppressing the action of $a_1{}^{m-1}$, and second by inducing a 'mutation' that allows the subsequent production of pigment. In all probability this 'mutation' is the transposition of the responding element away from A_1.

Both *Spm* and $a_1{}^{m-1}$ can undergo changes of state which markedly affect the phenotype. In the absence of a *Spm* element, $a_1{}^{m-1}$ behaves as a stable allele of A_1, but if *Spm* is present the $a_1{}^{m-1}$ complex can be modified so that it shows different degrees of $a_1{}^{m-1}$ activity and a different response to *Spm* (compare the states of $a_1{}^{m-1}$ in figure 17.4). The different states of $a_1{}^{m-1}$ differ in (i) the frequency with which the change from non-production to production of pigment occurs (the number of spots), (ii) the time during development at which these changes occur (the size of the spots), and (iii) the intensity of pigmentation. The range of possible phenotypes is further augmented by the occurrence of different states of *Spm*, each state modifying the expression of $a_1{}^{m-1}$ in a very precise manner; some changes of state of *Spm* reduce or abolish the ability of *Spm* to induce mutation in the $a_1{}^{m-1}$ complex (these modified *Spm* correspond to *Ac* elements that have lost their ability to induce the transposition of *Ds*), while other changes abolish both this mutation-inducing

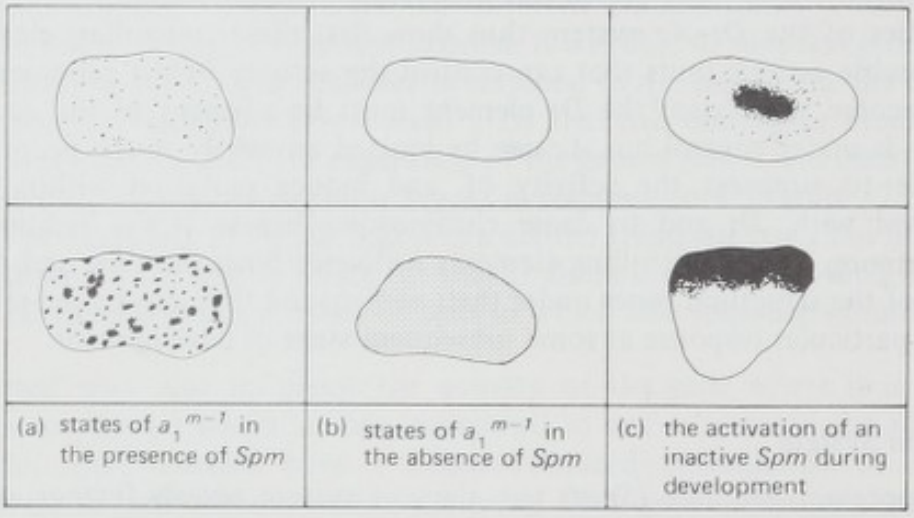


Figure 17.4 The *Spm*–$a_1{}^{m-1}$ system of controlling elements in maize.

(a) In the presence of the *Spm* activating element each mutation of $a_1{}^{m-1}$ to A_1 produces a coloured spot in the aleurone. The size and number of the spots is dependent on the particular state of $a_1{}^{m-1}$.

(b) In the absence of *Spm* mutations of $a_1{}^{m-1}$ to A_1 do not occur and the grains are either colourless or uniformly pigmented to varying degrees.

(c) These grains started development with an inactive *Spm* element. During the growth of the aleurone *Spm* was simultaneously activated in all the cells giving rise to the lower part of the kernels, so that in these cells the formation of pigment was suppressed.

response and also the ability of *Spm* to suppress $a_1{}^{m-1}$. These changes in *Spm* are not permanent, and subsequently *Spm* reverts to its original type of action—in other words, *Spm* undergoes *cycles of activity* from activity to inactivity and back again to activity. Each change of phase of activity regulates both the *time* and the *frequency* of occurrence of the return to the other phase; these changes may occur either frequently (every few cell generations) or infrequently (after many plant generations) but this always depends on the particular state of *Spm*. The two kernels shown in figure 17.4c started development with inactive *Spm* elements which were subsequently activated in the cells giving rise to the lower parts of the kernel so suppressing the formation of pigment in these cells. What is important is that from our knowledge of embryology we know that the colourless (activated *Spm*) regions could not have developed from one, or even from a few, reactivated cells—thus *Spm* must have been activated *simultaneously* in *many* different cells. This type of pattern is a common feature of developmental systems, and although, for the most part, we do not know how the cells are controlled to produce these patterns, the regular inactivation and activation of similar regulatory elements is a possible explanation.

Pre-setting Gene Activity

McClintock found several instances where *Spm* is able to pre-set the responding element-gene complex so that at a later stage in development the gene is expressed in a distinctive way. One complex that underwent pre-setting was $a_1{}^{m-2}$; when *Spm* is present, $a_1{}^{m-2}$ is activated and pigment is produced (this is in contrast to $a_1{}^{m-1}$, whose activity is suppressed by *Spm*) so that the kernels have a lightly pigmented aleurone with many small intensely pigmented spots. The pattern of the kernels without *Spm* depends on the past history of $a_1{}^{m-2}$. When a plant with a genotype $a_1/a_1{}^{m-2}$ and with one *Spm* element was crossed with an a_1/a_1 pollen parent with no *Spm* elements segregation produced ears bearing three types of kernel; 50 per cent of the kernels were a_1/a_1, and so were colourless; 25 per cent of the kernels were $a_1/a_1{}^{m-2}$ *Spm*, and were lightly pigmented with many intensely pigmented spots; and 25 per cent of the kernels were $a_1/a_1{}^{m-2}$ and had a curious mottled phenotype with the intensity of pigmentation varying from light to dark.

Kernels of each type were grown into mature plants and again crossed to an a_1/a_1 pollen parent. When the plants grown from the $a_1/a_1{}^{m-2}$ *Spm* kernels were crossed, exactly the same results were obtained, showing that the mottled phenotypes only occurred in the kernels that did not receive a *Spm* (in these crosses the $a_1{}^{m-2}$ complex could be followed because it was closely linked to the *Sh* shrunken allele). When the exceptional $a_1/a_1{}^{m-2}$ kernels were grown and crossed with an a_1/a_1 plant, most of the ears had only colourless kernels but some ears had a few kernels that still retained the mottled phenotype and, furthermore, the type and intensity of mottling was *always* exactly the same as the pattern on the grain that had been grown to produce the $a_1/a_1{}^{m-2}$ parent.

These results suggest that $a_1{}^{m-2}$ was *pre-set* by its earlier association with an active *Spm* so that at some later stage, probably just prior to the formation of the endosperm nucleus, it will respond in a particular way and produce a mottled phenotype. The results also suggest that the setting is normally erased at the following generation, probably at the same developmental stage, but that sometimes a particular setting escapes the erasure process and produces a mottled grain.

The importance of these results is that they show that a gene can be programmed at one stage in development so as to behave in a predetermined way at some later developmental stage—in other words it should be possible for a complete program of gene action to be imposed on a developing organism so that the genes are switched on only at the particular times during development when they are actually required.

At the molecular level we have little idea as to what controlling elements are or how they attach to or insert into the maize chromosome. Since they are stably inherited, they are probably DNA; this is borne out by cytological studies, which suggest that the controlling elements are sections of heterochromatin interspersed between regions of euchromatin. In bacteria we know that episomal DNA can be inserted into and excised from the bacterial chromosome by recombination, and it is tempting to speculate that controlling elements are also circles of DNA that can integrate into the maize chromosome at various positions—but as yet there is no evidence either for or against this hypothesis.

Controlling elements have been detected in many wild-type strains of maize and since they appear to be a normal component of the maize genome they should not be thought of as rather bizarre and unusual types of genetic element. Controlling elements have been detected in certain other organisms and they are thought to be the explanation of similar mutable systems in a variety of organisms from bacteria to mice.

X Chromosome Inactivation in Mammals

In both V-type position effects and controlling-element systems, the presence of heterochromatin affects the expression of adjacent euchromatic genes, possibly by heterochromatinisation spreading into the euchromatic genes and preventing their transcription; an even more striking example of this is the inactivation by heterochromatinisation of entire X chromosomes that occurs in female mammals. In man the cells from a normal female contain a small darkly staining body lying just within the nuclear membrane—this is the Barr body or the sex chromatin; the sex chromatin is absent from the cells of normal males, but in cells from individuals with abnormal sex chromosome constitutions it has been observed that there is always one less sex chromatin body than there are X chromosomes—thus an XXY male has one sex chromatin body, an XO female has none and an XXX female has two. This sex chromatin body is an X chromosome that has been inactivated by heterochromatinisation. In some mammals, such as mice, there is no sex chromatin body but one of the X

chromosomes assumes the appearance of heterochromatin and replicates precociously at cell division.

Following the recognition of the sex chromatin body as an X chromosome Mary Lyon developed the *inactive X* or *Lyon hypothesis*. According to this hypothesis one of the two X chromosomes of a normal female is irreversibly inactivated by heterochromatinisation about seven to twelve days after fertilisation. In some cells it is the maternal X and in others the paternal X chromosome that is inactivated, but the same X chromosome persistently remains inactivated in all the descendent cells.

Dosage Compensation

Although there are two X chromosomes in females and only one in males, the X-linked genes appear to function equally well in both sexes; the Lyon hypothesis offers a satisfactory explanation as to how this *dosage compensation* is achieved, because if one X in the female is always inactivated then, functionally, both male and female cells will have a single X chromosome. Several experimental results support this hypothesis. Firstly, the enzyme glucose-6-phosphate dehydrogenase in man is specified by a gene on the X chromosome (X^{Gd+}). It has been found that X^{Gd+}/Y males and X^{Gd+}/X^{Gd+} females have the same levels of enzyme activity (arbitrarily assigned the value of 100 per cent) while X^{Gd+}/X^{Gd-} females have only 50 per cent activity; the simplest explanation is that there is random X inactivation so that only half the cells that should produce the enzyme actually do so. The second and even more striking example is the dosage compensation seen in marmalade cats. Orange female cats are homozygous for an X-linked recessive gene (X^{b}) and if an orange

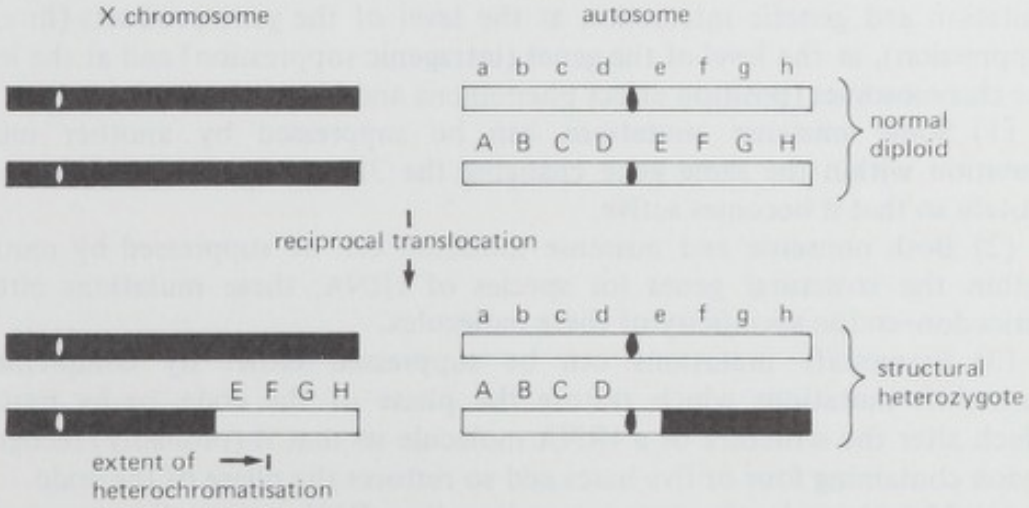


Figure 17.5 X-inactivation in the mouse. In a normal diploid cell (above) one X chromosome is inactivated but the genes on the autosomes are all expressed. Brian Cattenach observed that when autosomal genes are translocated on to an X chromosome (below) the genes nearest to the point of translocation (E and F) are inactivated together with the X chromosome, but the more distal genes (G and H) are expressed normally. The X chromosomes and the autosomes are represented in black and white respectively.

female (X^b/X^b) is crossed with a black male (X^B/Y) all the male progeny are X^b/Y and orange, and all the females are X^B/X^b; these females are *not* black as might be expected but have orange and black stripes. In some parts of the coat X^b is expressed and in other parts X^B depending on which X chromosome has been inactivated in that particular patch of coat–thus for certain genes the female mammal is a functional mosaic.

It seems very unlikely that *all* the genes on an inactivated X are non-functional. If a single X chromosome was all that was necessary for normal female development, XO and XXX individuals would be phenotypically alike–in fact, XO females are physically and sexually abnormal and have a condition known as Turner's syndrome. In a similar way, XXY males differ from XY males as they suffer from Klinefelter's syndrome. Furthermore, when a chromosome rearrangement has translocated autosomal genes onto an X chromosome, these genes tend to become inactivated in the same way as normal X-linked genes. A female heterozygous for such a translocation (figure 17.5) may be a mosaic for some of the characters determined by these autosomal genes (E and F) but not for characters determined by other genes (G and H); it appears that the nearer the autosomal gene to the point of translocation the greater is the probability that it will be inactivated. This is very similar to the V-type position effect phenomenon observed in *Drosophila* except that X-inactivation is irreversible while position-effect inactivation is not.

Summary of Key Points

In this chapter we have considered some further and particular aspects of mutation and genetic interaction at the level of the gene products (intergenic suppression), at the level of the genes (intragenic suppression) and at the level of the chromosomes (position effect phenomena and X-inactivation).

(1) Some missense mutations can be suppressed by another missense mutation within the same gene changing the 3D configuration of the inactive protein so that it becomes active.

(2) Both nonsense and missense mutation can be suppressed by mutations within the structural genes for species of tRNA; these mutations alter the anticodon-codon specificity of these molecules.

(3) Frameshift mutations can be suppressed either by complementary frameshift mutations which restore the phase of the code, or by mutations which alter the structure of a tRNA molecule so that it (probably) recognises a codon containing four or five bases and so restores the phase of the code.

(4) Mutations in the genes encoding the rRNA components occasionally result in the misreading of the genetic code during translation.

(5) When a gene normally located in euchromatin is moved to a new position near heterochromatin, it can become heterochromatinised and inactivated to a variable extent (V-type position effect); the nearer the gene to heterochromatin, the more frequently it is inactivated.

(6) Controlling elements are autonomous regulatory elements which act to control the activity of structural genes. A two-element system consists of a responding element located adjacent to the structural gene being regulated and an unlinked signalling element which controls the activity of the responding element.

(7) Controlling elements can (i) induce mutation and chromosomal breaks at the point where they are inserted into the chromosome, (ii) partially or completely suppress gene activity, (iii) transpose from one location in the genome to another, (iv) be modified so that their ability to mutate, suppress or transpose is altered, and (v) be pre-set so that at a subsequent stage in development they will behave in a predetermined way.

(8) In female mammals one of the two X chromosomes is inactivated by heterochromatinisation, and most of the genes on this X chromosome will be inactivated. This is a mechanism for block regulation by which whole groups of genes can be coordinately inactivated.

18 Genetic Organisation and Function in Eucaryotes

Wonders are many, and none is more wonderful than man.
Sophocles (495–406 BC)

The preceding chapters have, for the most part, examined the organisation and regulation of the genes in viral and bacterial systems; in this chapter we shall discuss some particular aspects of genetic regulation in higher organisms and summarise our understanding of how their DNA is organised. In the comparatively simple systems of bacteria and viruses we know a great deal about the ultimate structure of some genes and the molecular basis of the regulation of their activity. Although it is clear that highly specific control systems must also exist in eucaryotes, we do not generally know just how similar these are to the bacterial control systems, and except in a few special instances there is no evidence that eucaryotic genes are organised into operons like those found in bacteria.

After an egg has been fertilised the zygote undergoes repeated mitoses and eventually develops into an adult organism. The most striking feature of this developmental process is that the *genetically identical* daughter cells *differentiate* into many *phenotypically different* cell types, such as nerve cells, muscle cells, liver cells, erythroblasts and so on, and in recent years molecular geneticists and developmental biologists have expended considerable efforts in attempts to elucidate the molecular basis of cell differentiation.

Differential Gene Activity

The variations between separately differentiated cells are due largely to differences in their protein compositions; thus insulin is made only in special cells in the pancreas, while tyrosinase is made only in the melanocytes. Since these proteins are specified by genes, the way a particular cell differentiates must depend on the particular genes that are active in that cell; this does not mean that in a particular type of differentiated cell certain genes are active all the time, but rather that the range of genes that can be activated and inactivated is restricted. Thus gene activity will vary enormously not only from one cell type to another but also in the same cell type at different stages of development. It is important to note that what we actually observe in differentiated cells is differential protein synthesis and not differential gene activity; protein synthesis can be controlled not only by regulating the rate of transcription (true differential gene activity) but also by regulating the differential replication of selected genes and by regulating the rate of translation. The term differential gene activity is commonly used, albeit incorrectly, to refer to the control of

protein synthesis at all possible levels. Three examples will be described to illustrate this concept of differential gene activity, the central thesis of developmental genetics.

(1) At least six non-allelic genes (*Hbα*, *Hbβ*, *Hbγ*^{1}, *Hbγ*^{2}, *Hbδ* and *Hbϵ*) are involved in the synthesis of the human haemoglobins, and although *Hbα* is active at all times, the other genes are active at different times during development (table 18.1). In adults the main haemoglobin is HbA, a tetramer made up of two

Table 18.1 Differential activity of the genes involved in the production of the human haemoglobins

Developmental stage	Genes active						Haemoglobins present
	Hbα	*Hbβ*	*Hbγ*^{1}	*Hbγ*^{2}	*Hbδ*	*Hbϵ*	
early foetus	+++			+		+++	E_1 ϵ_4 E_2 $\alpha_2\epsilon_2$ E_3 $\gamma^2\epsilon_2$
neo-natal foetus	+++		++	++			F_1 $\alpha_2\gamma_2^1$ F_2 $\alpha_2\gamma_2^2$
late foetus and adult	+++	+++			+		A $\alpha_2\beta_2$ A_2 $\alpha_2\delta_2$

alpha and two beta polypeptide chains, and to a lesser extent (2.5 per cent) there occurs haemoglobin HbA_2 made up of two alpha and two delta polypeptides—these three polypeptides are specified by the *Hbα*, *Hbβ* and *Hbδ* genes respectively. However, in the early foetus, the related haemoglobins HbE_1, HbE_2 and HbE_3 are found instead of HbA. These haemoglobins consist of four epsilon chains, two alpha and two epsilon chains, and two gamma^2 and two epsilon chains respectively, showing that the *Hbα*, *Hbγ*^{2} and *Hbϵ* genes are active. In the neo-natal embryo the two haemoglobins present are HbF_1 and HbF_2, made up of two alpha and two gamma^1 chains or two alpha and two gamma^2 chains—thus *Hbγ*^{2} has been inactivated and *Hbγ*^{1} activated. The final change occurs at about the twentieth week, when *Hbγ*^{1} and *Hbγ*^{2} are inactivated and *Hbβ* and *Hbδ* are activated.

(2) In the giant polytene chromosomes of the Diptera, the presence of a *puff* (figure 18.1) is a visual indication of the activity of the genes located in the band that is puffing. For example, in the left arm of chromosome III of *Drosophila* there is a very precise pattern of puffing in the salivary gland cells; there is a period of intense activity preceding puparium formation and another period of activity approaching the pupal moult (figure 18.2). Although some bands are active in both periods other bands (75CD and 78D) are active in only one period or the other.

(3) Lactic dehydrogenase is an indispensible enzyme that is apparently found in all vertebrate cells, and it enables them to function during periods of

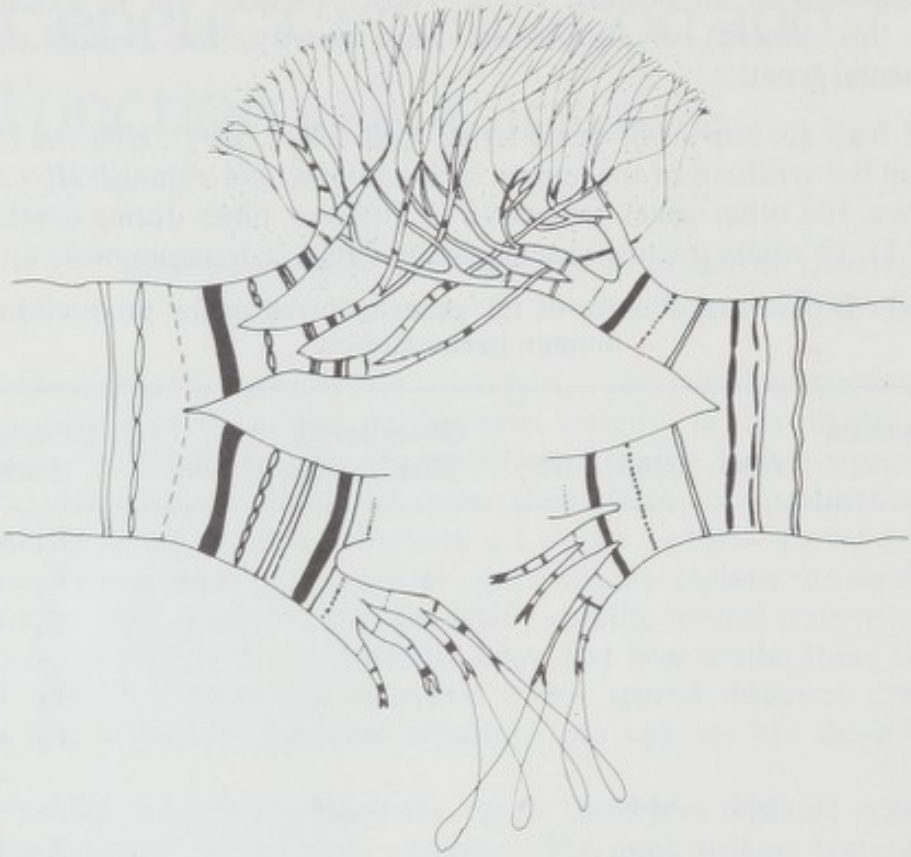


Figure 18.1 Puffing in a polytene chromosome. Schematic representation of a large puff as seen under the light microscope (above) showing how the polytene chromosome is progressively split into smaller and smaller sub-units with the result that the genetic material within the puff is greatly extended and available for active transcription. It is inferred from electron micrographs (below) that the individual chromatids are continuous from one side of the puff to the other. Only a few of the very many chromatids are shown.

temporary oxygen shortage. The enzyme can be separated from cell homogenates by starch-gel electrophoresis, a technique that separates macromolecules according to their net electrical charge and shape. The homogenate is inserted into a block of an inert starch gel and a voltage applied across the gel; each different species of macromolecule separates into a band across the gel, the position of the band depending on the net charge and shape of the molecules. The bands containing particular enzyme molecules can be identified by staining the gel with a histochemical stain specific for the type of enzyme being isolated. After electrophoresis lactic dehydrogenase (LDH) is located in five separate bands, each band containing a different macromolecular form of the enzyme; each of these *isozymes* is a tetramer made up of two different types of polypeptide subunits, A and B, each with a molecular weight of about 35 000. The five bands observed after electrophoresis contain the five possible combinations of subunits, B_4, B_3A, B_2A_2, BA_3 and A_4, known as LDH-1 to LDH-5 respectively (table 18.2). The amounts of each isozyme present indicate the relative numbers of A and B subunits that were present in the cells and so is a measure of the relative activities of the *A* and *B* genes. What is interesting is

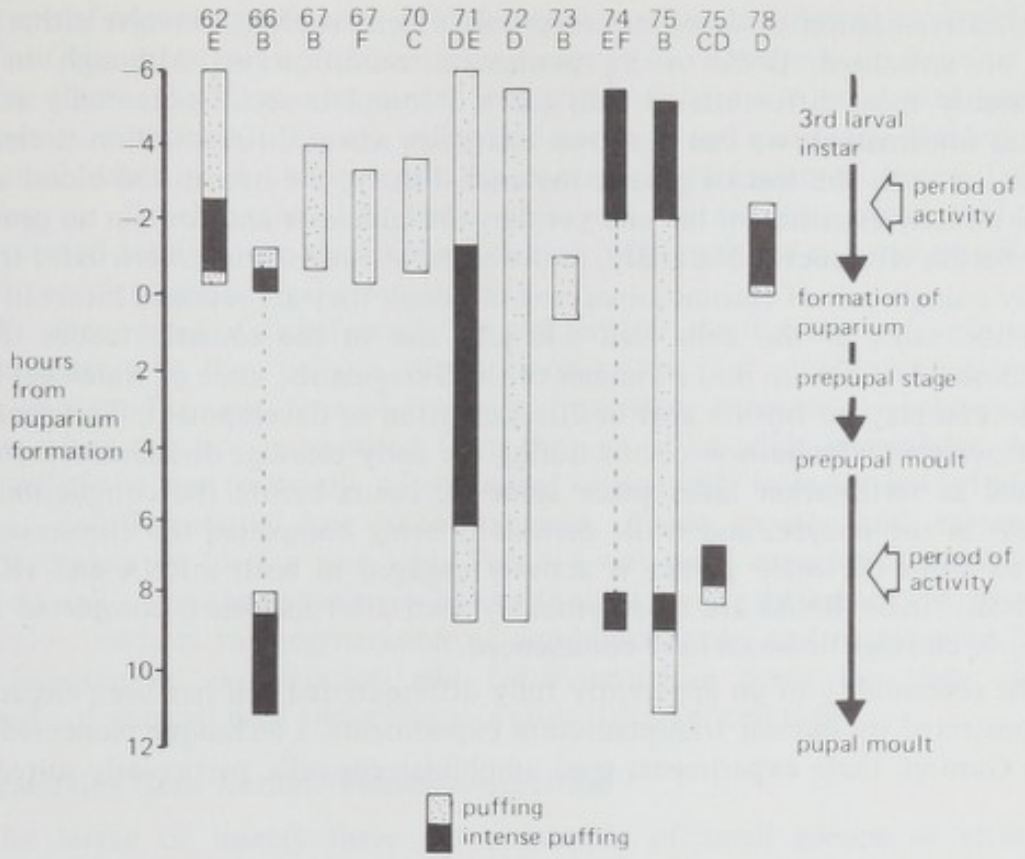


Figure 18.2 Differential gene activity in *Drosophila*. The pattern of puffing in the left arm of chromosome III during the prepupal stages. Each particular band (identified by a number and letter) exhibits puffing at a characteristic stage in development; some bands are active only in the period before the formation of the puparium (band 62E for example), some only in the period leading up to the pupal moult (bands 75C and D) and others in both periods (bands 74E and F).

that different isozymes are present not only in different species but also in different cells of the same species. In the egg cells of mice only LDH-1 (B_4) is present, so that *B* is very active and *A* is inactive. By about the ninth day of development the distribution is skewed towards LDH-5 so that *A* is now more active than *B*, while still later in development *B* is progressively reactivated shifting the distribution back towards LDH-1. These differences involve not only the cyclic activation and inactivation of *A* and *B* but also the differential rates of protein synthesis *after* activation. It is even more striking that the spermatogonia of mammals and birds contain A and B subunits, showing the activity of *A* and *B*, while in the spermatocytes, which are derived from the spermatogonia, *A* and *B* are suppressed and another gene, *C*, is activated; the *C* subunits are only produced for a few hours and then *C* is switched off until the next generation.

These examples suggest that (i) a particular protein can only be synthesised by a cell after the corresponding gene has been activated, and (ii) even after activation the *rate* of gene activity can be very precisely controlled.

Differentiation is Reversible

We do not know how the genes are activated and inactivated in this way, but

since differentiation is sometimes reversible it cannot always involve either the loss of unwanted DNA or its permanent modification. Although in all probability most differentiated cells carry a complete set of potentially active genetic information, we can note two examples where differentiation is clearly associated with the loss of genetic material. Firstly, the human red blood cells are absolutely essential for life and yet they are anucleate and contain no genetic information whatsoever. Secondly, in the roundworm *Ascaris lumbricoides* there is only a single pair of chromosomes, and although they are retained intact in the germ-line cells, in the cells that will give rise to the somatic tissues these chromosomes break up into a number of small fragments, some of which get lost and so can play no further part in differentiation or development. This process of *chromosome diminution* occurs during the early cleavage divisions. *Ascaris* is unusual as fertilisation takes place some 60 hours before the completion of meiosis in the oocyte, and while meiosis is being completed the chromosome derived from the male parent is actively engaged in both mRNA and rRNA synthesis; these RNAs are most probably used after meiosis is completed and the early cleavage divisions have commenced.

The reversibility of an apparently fully differentiated cell has been elegantly demonstrated by nuclear transplantation experiments, a technique pioneered by John Gurdon; these experiments used amphibian egg cells, particularly suited to

Table 18.2 Differential activity of the lactic dehydrogenase *A* and *B* genes

	Relative gene activity		Designation and composition of isozymes				
	A	*B*	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
			BB BB	BB BA	BB AA	BA AA	AA AA
egg cells	–	++++	++++	–	–	–	–
9-day cells	+++	+	–	–	–	+	++++
at birth	++	++	+	++	++++	++	+
adult	+++	+	–	+	++	++++	++

The enzyme LDH, extensively studied by Clement Markert, is a tetramer made up from the A and B subunits specified by the *A* and *B* genes. At different stages of development there are different amounts present of the five possible isozymes, showing the differential activity of the *A* and *B* genes.

The + and – symbols indicate the relative activities of the *A* and *B* genes (left-hand columns) and the relative amounts of each isozyme present (right-hand columns) in mouse cells at different stages of development.

this type of experiment because of their very large size. If a diploid nucleus from a cell of the intestinal epithelium of a *Xenopus laevis* tadpole (*X. laevis* is the African Clawed Toad) is transplanted into an unfertilised and enucleated egg cell then, just occasionally, this egg will divide normally and eventually develop into a normal adult toad (other egg cells containing transplanted nuclei fail to develop or develop into abnormal embryos). It is quite clear that the transplanted nucleus can dedifferentiate and redifferentiate so as to be able to direct complete and normal development.

The phenomena of dedifferentiation and redifferentiation are not confined to the laboratory as they occur naturally in organisms that are able to regenerate new limbs and organs. One of the most interesting examples of regeneration occurs when the lens is removed from the eye of an adult salamander. After removal of the lens (normally of epidermal origin) cells from the iris (of neural origin) dedifferentiate, lose their pigment, divide rapidly and eventually redifferentiate to form a new lens. This is a very good example of *metaplasia*, where a cell of one type has given rise to a cell of a quite different type. In most examples, such as the regeneration of amphibian limbs and mammalian liver, dedifferentiation, proliferation and redifferentiation occur but there is no evidence of one cell type giving rise to a quite different cell type.

Programming Gene Action–Transdetermination

In the larvae of insects there are a number of small groups of virtually undifferentiated cells with large nuclei and containing many free ribosomes; these are the *imaginal discs*. During metamorphosis, when the change from larva to adult occurs inside a puparium, these imaginal discs differentiate to produce adult structures–in *Drosophila* there are six leg discs and each produces a leg, two wing discs produce the wings, a genital disc the genital structures, and so on. If a genital disc is removed from a larva and cut up, and the pieces are implanted into a host larva of the same age, then each piece will, in the main, give rise to just one particular structure within the complex of the genital organs. Thus although the cells of the imaginal discs are as yet undifferentiated, each disc cell is clearly *programmed* (determined) *so as to develop in a particular way*. In spite of this rigid programming the disc cells can be reprogrammed so as to develop into a different structure (figure 18.3). If, for example, cells from a male genital disc are implanted into an *adult fly* the cells will proliferate and grow into a larger than normal disc–cells from this disc can now be implanted into another adult fly and by serial cultures the disc cell line can be maintained indefinitely. When pieces of one of these discs are implanted into host *larvae* they will normally grow into the genital structures specified by the cells in the original disc, but just occasionally, and after many cell divisions in the adult fly hosts, the original program is lost and the cells are reprogrammed; the implanted disc can now develop into a leg or antenna, structures that are normally determined by other discs. The original disc cells have undergone *transdetermination* and will retain their new program indefinitely.

There can be no doubt that programming mechanisms are a normal part of the developmental process, and their importance in ensuring the correct

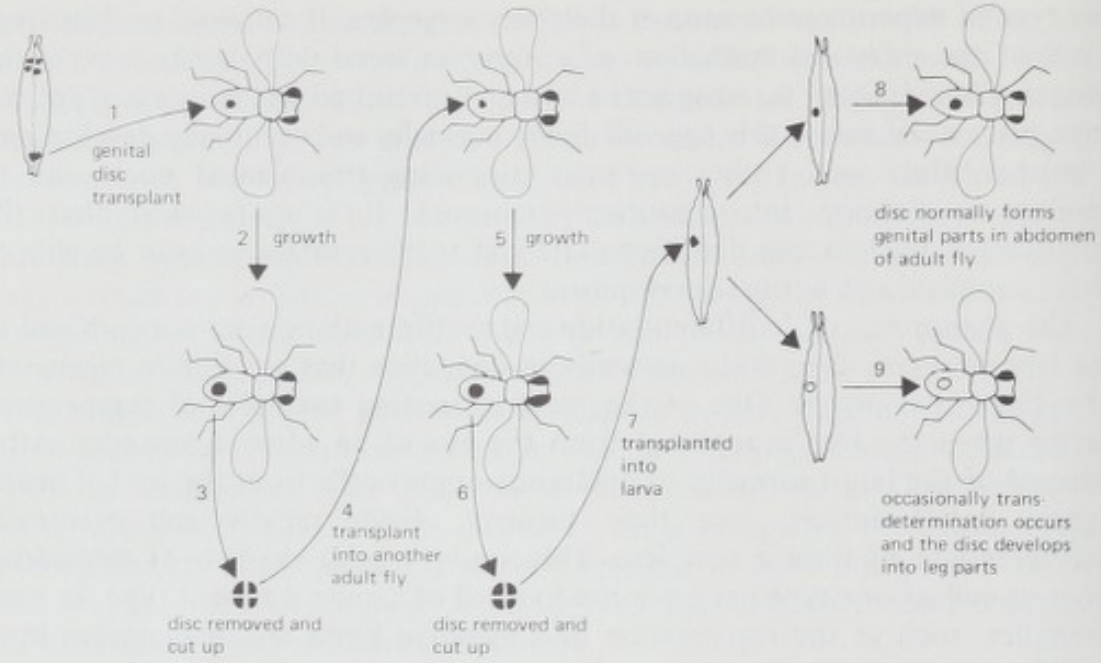


Figure 18.3 Transdetermination. The figure illustrates one of Ernst Hadorn's experiments on transdetermination in *Drosophila*.

(1) An undifferentiated genital disc is transplanted from a larva into the abdomen of an adult fly.

(2) The genital disc cells proliferate but do not differentiate.

(3) The disc is removed and cut up into pieces.

(4) One piece is transplanted into another adult fly.

(5) The disc cells proliferate.

(6) The foregoing procedure is repeated very many times.

(7) Many generations later, pieces from the disc cells are transplanted into larvae.

(8) After metamorphosis most of the disc implants are found to have developed into genital parts, floating free in the abdomen of the adult fly.

(9) Occasionally one of the implanted fragments develops into leg parts–the original disc cells have undergone transdetermination.

sequence of events cannot be overemphasised. When imaginal disc cells are transplanted from *Drosophila* larvae into adult flies the disc cells proliferate but never develop into organs—this only occurs at metamorphosis so that some external stimulus, probably one of the hormones produced at metamorphosis, is necessary to stimulate the activity of the imaginal disc cells. It is also evident that what determines the particular disc cells that are activated is the way they have been programmed; in different disc cells, alternative sets of genes have been pre-set so as to be activated in response to the stimulus. Just how this programming is achieved is quite unknown, and the pre-setting of maize genes by controlling elements remains the only instance where the programming is set by identifiable genetic components.

The Control of Differential Gene Activity

We do not know how eucaryotic genes are switched on and off, but it is evident that the amounts and types of gene products present in the cell can be regulated

by controlling (i) differential gene replication, (ii) the rate of transcription, and (iii) the rate of translation. The control of the rates of transcription and translation in procaryotic systems has already been described and is not a new concept, but the idea of differential gene replication is that selected genes are replicated so that they are present in many copies and so will contribute more gene product than will a single unreplicated gene.

Differential Gene Replication

In higher organisms the genes encoding the 18*S* and 28*S* rRNAs are located in the nucleolar organiser region and in both *Xenopus* and *Drosophila* abnormal individuals with no nucleolar organisers are quite unable to synthesise rRNA and they die during their early development. What is so remarkable is that each nucleolar organiser region contains not just one copy of each rRNA gene but very many copies in an alternating tandem array (page 331), providing some of the best-known examples of differential gene replication. In *Xenopus* each nucleolar organiser region contains about 450 18*S* and 450 28*S* rRNA genes; the genes for the 5*S* rRNA are not located in the nucleolar organiser region, but over 24 000 copies of this gene are arranged in groups dispersed throughout the genome. In *Drosophila* there are about 100 copies and in *Nicotiana tabacum* about 750 copies of each of the 18*S* and 28*S* rRNA genes. Even in bacteria there appear to be multiple copies of the genes for some rRNAs; in *Bacillus subtilis* there are about ten copies of the 16*S* and 23*S* rRNA genes and about four copies of the 5*S* rRNA gene. This lateral multiplicity of the rRNA genes is found in every cell of the individuals, irrespective of the actual rates of rRNA synthesis, and it is obviously a mechanism to allow for the rapid synthesis of quite large amounts of rRNA. However, this is not the end of the story, because in amphibian oocytes, where at certain developmental stages there is an *extremely* high rate of rRNA synthesis, there is a further *amplification* of the 18*S* and 28*S* rRNA genes. Each oocyte nucleus has not four (the oocyte is effectively a tetraploid cell) but many hundreds of nucleoli each containing many further copies of these rRNA genes; each oocyte may contain up to 1000 times the number of copies of the 18*S* and 28*S* rRNA genes found in a somatic cell. The 5*S* rRNA genes are not amplified in this way, and this may be the reason why there are so many copies present in the somatic cells. Note that these nucleoli and the ribosomal genes they contain are completely free of the chromosomes, so that this DNA is an important exception to the general premise that the bulk of the DNA is located in the chromosomes.

We conclude that the very high rate of rRNA synthesis in the oocytes is due to the genes concerned being present in very many copies, while variations in the rate of rRNA synthesis in the somatic cells must be due to control at the level of either transcription or translation.

Transcriptional Control

Control at the level of transcription is universal and is probably the most important way of regulating gene activity. In procaryotes this is achieved

primarily by specific protein repressor molecules binding to particular nucleotide sequences along the chromosomes and preventing the initiation of transcription by RNA polymerase; however, we cannot be sure that similar systems of control exist in higher organisms. The chromosomal DNA in higher organisms exists as a highly complex nucleo-histone containing both histone and non-histone proteins. Because (i) there are relatively few species of histones, (ii) their composition is very similar from one organism to another, and (iii) their content remains very similar throughout all stages of development, it is unlikely that the histones play an important part in controlling the activity of *specific* genes. On the other hand, the non-histone proteins vary greatly, both in type and amount, from one tissue to another, and the available evidence suggests that they are in some way involved in the control of transcription.

There is no doubt that transcription can be regulated by cytoplasmic factors. For example, when the nucleus from a differentiated amphibian cell that is rapidly synthesising ribosomal RNA is transplanted into an enucleate egg the synthesis of rRNA immediately stops; the egg cells do not normally synthesise rRNA and their cytoplasm must contain some substance which can enter the transplanted nucleus and inactivate the rRNA genes. We know that there is the regular movement of proteins between the cytoplasm and the nucleus, and although some of these remain bound in the nucleus and are probably structural proteins of one kind or another, others move in both directions and could be responsible for the regulation of specific gene activity. Gene action can also be regulated by hormones. During dipteran metamorphosis the larval moult will not occur unless the hormone ecdysone is present, and when ecdysone is injected into larvae the first visible change is the puffing of certain bands in the salivary gland chromosomes. In rat liver cells the hormone hydrocortisone stimulates the increased synthesis of the enzyme tryptophan pyrrolase. Even more important, the hormonal induction of protein synthesis does not occur if actinomycin is present; this antibiotic prevents transcription by complexing with the DNA template, strongly suggesting that hormones act by controlling transcription. It is tempting to speculate that hormones act in the same way as effectors in procaryotic systems, but it cannot be ruled out that they act indirectly by causing intracellular changes which in turn affect transcription.

Heterochromatinisation is also able to influence the rate of transcription. The DNA in heterochromatin remains tightly compacted during interphase while the DNA in euchromatin uncoils and becomes dispersed. All the evidence indicates that the heterochromatic regions are transcriptionally inactive, probably because the coiling prevents the DNA from being accessible as a template for transcription; it is likely that regions that are normally (constitutively) heterochromatic are devoid of any genes. The V-type position effect phenomena found in *Drosophila* and the inactivation of mammalian X chromosomes (chapter 17) are examples of transcriptional control by the heterochromatinisation of DNA that is normally euchromatic. This facultative heterochromatinisation is normally a reversible condition, itself under cytoplasmic control.

Translational Control

The rate at which a gene is transcribed is only one of the factors limiting the amount of gene product that can be produced; another is the number of times that a given molecule of mRNA can be used as a template and this, in turn, depends on the time that the mRNA acts as a stable template. In bacteria, most mRNAs are very-short lived and have a half life of only two to five minutes, but in eucaryotes a typical short-lived mRNA has a half life of about two hours. Some higher organism mRNAs may have a very long life, but for most of this time they are stored in an inactive form and cannot be used as templates for translation. The longest-lived mRNAs function as informational store houses because they can be accumulated over a long period of time and released when a burst of activity is required after a period of quiescence. For example, in germinating seeds polyribosomes are formed and protein synthesis initiated even in the absence of RNA synthesis; thus the seed must contain a store of stable mRNA templates. Lotus seeds are still able to germinate after 1700 years, so their messengers can survive for very long periods indeed. A similar situation is

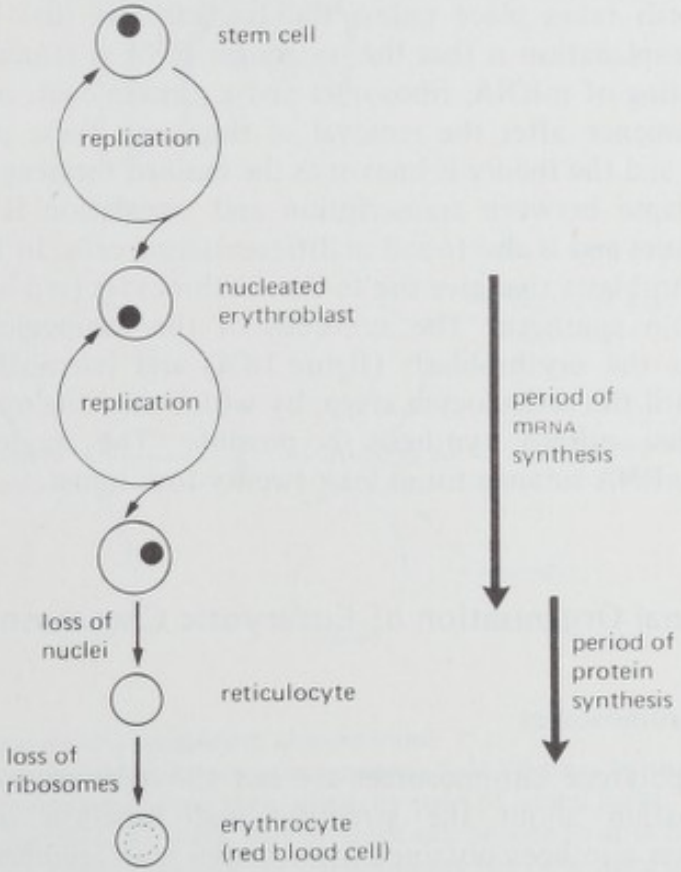


Figure 18.4 The development of erythrocytes in man. A population of dividing stem cells gives rise to more stem cells which differentiate into nucleated erythroblasts; these continue to divide until they differentiate into reticulocytes by the loss of their nuclei. Finally, the reticulocytes lose their ribosomes and become erythrocytes (red blood cells). mRNA is synthesised only in the erythroblasts, while haemoglobins and other proteins are made only in the reticulocytes.

found in animal eggs. Immediately after the fertilisation of a sea-urchin egg there is a considerable increase in the rate of protein synthesis and most of these proteins are synthesised even in the presence of actinomycin. The obvious explanation is that post-fertilisation protein synthesis does not require the synthesis of mRNA because the necessary templates already exist in the cytoplasm of the egg and are only released after fertilisation. In contrast, the extensive protein synthesis that occurs at gastrulation does not take place if actinomycin is present; thus most mRNAs are not transcribed until the onset of gastrulation. (Certain post-fertilisation proteins are absent from actinomycin-treated embryos, so that a few specific mRNAs are probably synthesised immediately following fertilisation.)

The unfertilised egg carries a store of suppressed maternal informational (messenger) RNA which is activated following fertilisation. What is the mechanism that prevents this mRNA from being transcribed in the unfertilised egg, and what is the nature of the change that results in activation? A fraction can be isolated from sea-urchin eggs that contains both ribosomes and informational RNA, but when this fraction is used in an *in vitro* system no protein synthesis takes place unless the fraction has first been treated with trypsin. One explanation is that the messenger RNA is stored in a cytoplasmic particle consisting of mRNA, ribosomes and a protein coat, and that translation can only commence after the removal of this coat; these particles are called *informosomes* and the theory is known as the masked messenger theory.

The time lapse between transcription and translation is not restricted to embryonic tissues and is also found in differentiating cells. In the nucleated stem cells and erythroblasts that give rise to the erythrocytes (red blood cells) there is no haemoglobin synthesis. The synthesis of the haemoglobin mRNAs only commences in the erythroblasts (figure 18.4) and haemoglobin itself is not synthesised until the reticulocyte stage, by which time the nuclei have been lost and no further mRNA synthesis is possible. The moderately short-lived haemoglobin mRNA survives for at least twenty-four hours.

The Functional Organisation of Eucaryotic Chromosomes

Lampbrush Chromosomes

The dipteran polytene chromosomes are not the only giant chromosomes and useful information about the structure and function of the eucaryotic chromosome has also been obtained from studies with *lampbrush chromosomes*, a special type of chromosome found in the oocytes of many vertebrates and of some invertebrates. These chromosomes, so called because of their resemblance to an old-fashioned lampbrush, are only found during the mid-prophase of meiosis, after chiasmata have formed but before the chromosomes condense. At this stage, which can last up to twelve months, the oocyte matures and synthesises most of the very large quantity of RNA required for the

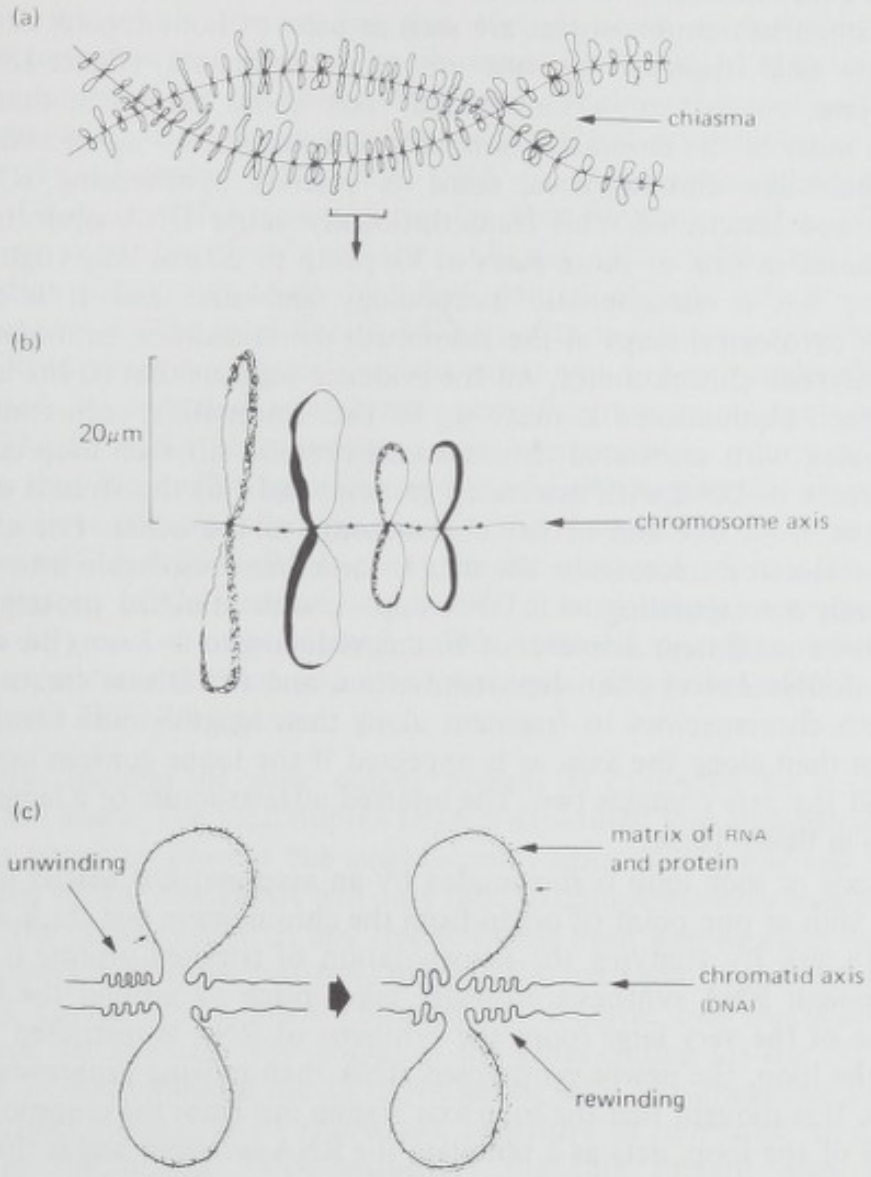


Figure 18.5 The structure of a lampbrush chromosome.

(a) A pair of homologous lampbrush chromosomes held together by two chiasmata. The chromosomes are differentiated along their length by pairs of lateral loops.

(b) At a higher magnification each pair of loops can be seen to arise from an individual chromomere; there are many hundreds of chromomeres but only some give rise to loops. Each loop is surrounded by a markedly asymmetric matrix of RNA and protein.

(c) The inferred structure of a loop. It is thought that the longitudinal axis of the chromosome consists of two individual chromatids, each made up from a molecule of duplex DNA with attached protein, and that the chromatids are continuous around the loops. The loop appears to be spun out from the chromomere at its thin end and rewound into the chromomere at the other end – thus a molecule of RNA transcribed at the thin end of the loop (represented by the small arrow) will move progressively around the loop.

post-fertilisation cleavage divisions. In order to achieve this, the parts of the chromosome that are actively involved in RNA synthesis are relatively uncondensed and their DNA is uncoiled; in frog and newt oocytes these extended lampbrush chromosomes may be nearly 1 mm long.

The lampbrush chromosomes are seen as pairs of homologous chromosomes (bivalents) held together by one or more chiasmata (figure 18.5a). Each chromosome consists of a longitudinal axis about 50 nm in diameter, and although most of the chromatin is tightly compacted into dense and irregularly spaced bead-like chromomeres, some is actively synthesising RNA and is uncoiled and functional. This transcriptionally active DNA projects from the chromomeres as one or more *pairs* of loops up to 20 μm long (figure 18.4b)—each loop has a characteristic morphology and size, and it is possible to construct cytological maps of the lampbrush chromosomes, equivalent to those of the polytene chromosomes. All the evidence suggests that (i) the longitudinal axis of each chromosome is made up of two chromatids, each consisting of a DNA duplex with associated chromosomal protein; (ii) each loop consists of a single duplex of DNA with associated protein; and (iii) the strands of DNA are continuous from one end of the chromosome to the other. For example, (i) under the electron microscope the axis is sometimes resolvable into two 15 nm fibres, each corresponding to a DNA duplex with attached protein, while the loops have a maximum diameter of 10 nm, reducing to 2–3 nm (the diameter of a DNA double helix) after deproteinisation; and (ii) DNase treatment causes lampbrush chromosomes to fragment along their length—more breaks occur in the loops than along the axis, as is expected if the loops contain one strand of DNA and the axis contains two. The inferred ultrastructure of a lampbrush loop is shown in figure 18.5c.

The axis of each loop is surrounded by an asymmetrical matrix of RNA and protein, thin at one point of origin from the chromomere and thick at the other (figure 18.5b). By studying the incorporation of tritiated uridine it was found that although RNA synthesis normally takes place all around the loops, in at least one of the very large loops the synthesis of RNA is restricted to the thin end of the loop, the newly synthesised RNA then moving progressively around the loop. It is thought that the loop axis is spun out from the chromomere at the thin end of the loop, acts as a template for RNA synthesis and is then rewound into the chromomere at the thick end of the loop. Each chromomere, like a band in a polytene chromosome, is sometimes thought to represent only a single gene or a closely related group of genes, and yet each loop contains enough DNA to accommodate many hundreds of genes; one explanation of this apparent paradox is that the genetic information is *serially replicated* around the loop, the multiple gene copies enabling the more rapid synthesis of the RNAs required.

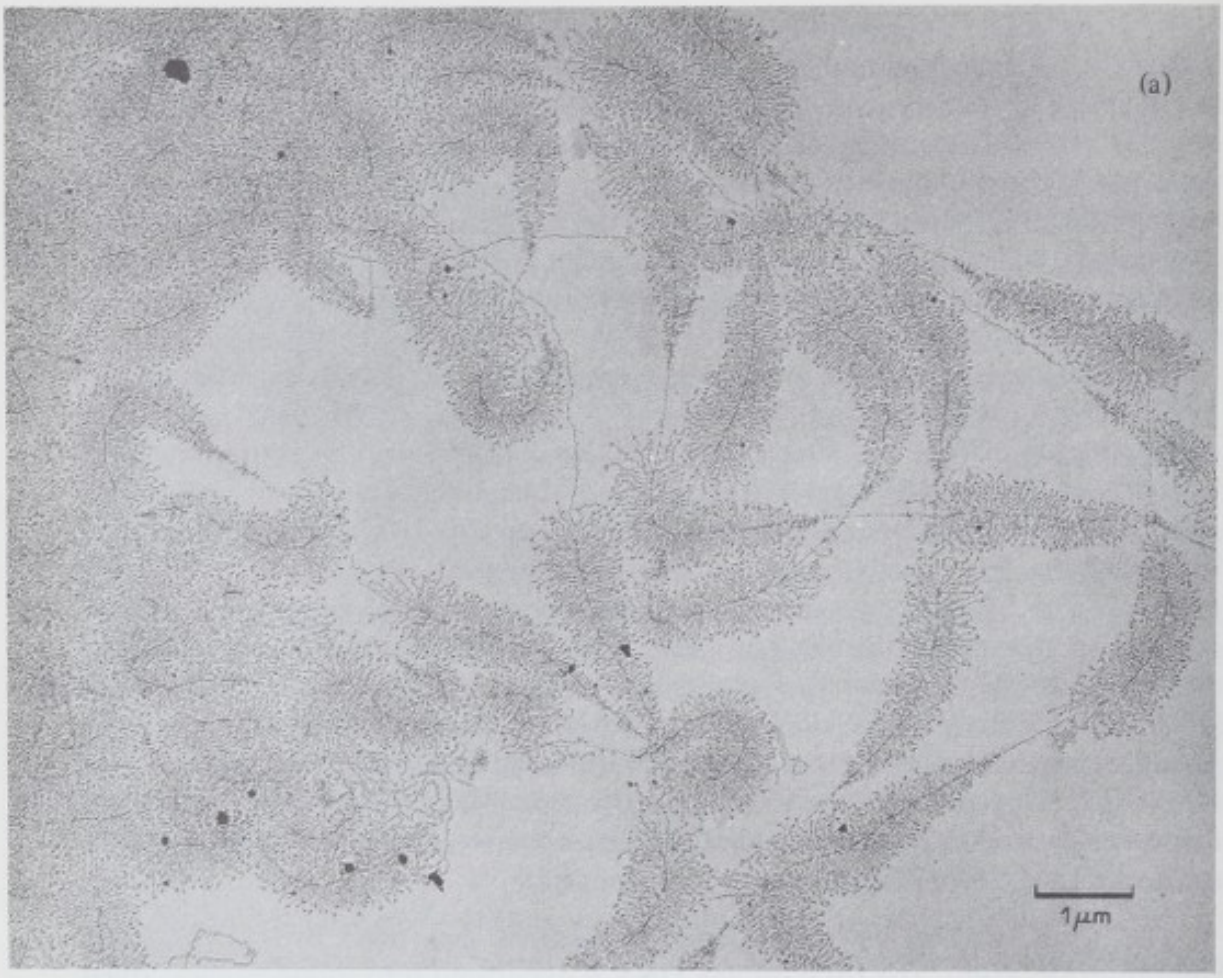
The Ribosomal RNA Genes of *Xenopus*

In chapter 4 we implicitly assumed that the eucaryotic chromosome always carries a unique linear sequence of genes, each gene being present once and once

only; it is now abundantly clear that this is not always so. Important information about how multiple gene copies are arranged along the chromosome and how they function has been obtained from studies with the ribosomal RNA genes of the African Clawed Toad, *Xenopus laevis* (see page 325). This is a particularly favourable system for this sort of study as not only are the immediate gene products (that is, the ribosomal RNA, rRNA) easily isolated but very many copies of the ribosomal RNA genes are present in each cell. The first achievement using this system was the isolation and purification of the genes specifying the ribosomal RNA (the DNA of these genes is referred to as ribosomal DNA, or rDNA), carried out by Max Birnstiel and his colleagues in 1967. They used the fact that the 18*S* and 28*S* rRNA, and hence the corresponding rDNA, has a very high G-C content (53-63 per cent, compared with 40 per cent for the total *Xenopus* DNA); thus molecules containing the 18*S* and 28*S* rRNA genes are denser than the other and A-T rich molecules and can be separated by repeated equilibrium centrifugation in a caesium chloride gradient. The 5*S* RNA genes, however, are interspersed in regions of high A-T content so that the molecules containing the 5*S* rRNA genes are unusually *light* and band above the so-called mainband DNA containing the non-ribosomal DNA. The 18*S* and 28*S* rRNA genes are all clustered within the nucleolar organiser regions of the genome and so are isolated on the *same* molecule of DNA—the 5*S* genes are not located in the nucleolar organiser region but are dispersed around the genome and so do not occur on the same DNA molecules as the 18*S* and 28*S* rRNA genes.

The total number of gene copies present is estimated by a DNA/RNA *hybridisation assay*. The total duplex DNA is denatured into single strands, fixed on a filter paper to prevent the single strands from reannealing and incubated with labelled rRNA. The rRNA will only bind to complementary base sequences, that is, to the rDNA, and after any free RNA has been washed away the amount of radioactivity will show just how much RNA has been bound and hence the number of copies in the DNA complement. It turns out that each haploid chromosome set contains 450 copies of the genes coding for the 18*S* and 28*S* rRNA and 24 000 copies of the 5*S* rRNA genes. DNA sequences that are present in many copies are said to be *redundant, repetitive* or *reiterated,* to distinguish them from the *unique* DNA sequences that are present only once per chromosome set.

In 1969 Oscar Miller isolated functioning rDNA from living oocyte nuclei in the spotted newt, *Triturus viridescens,* and examined it by electron microscopy. Among the nucleolar DNA he observed circular DNA molecules consisting of an axial fibre 10-20 nm in diameter periodically covered by regularly repeating matrix material (figure 18.6); within each matrix unit 80-100 matrix fibrils were attached by one end to the core axis, and there was a gradient of fibril length from one end of the matrix unit to the other. Since the axial fibre could only be broken by DNase and the matrix fibrils only by RNase, Miller concluded that each fibril was the RNA product of transcription and that the regularly repeating patterns occurred wherever transcription was active; he also observed that


(a)
1μm

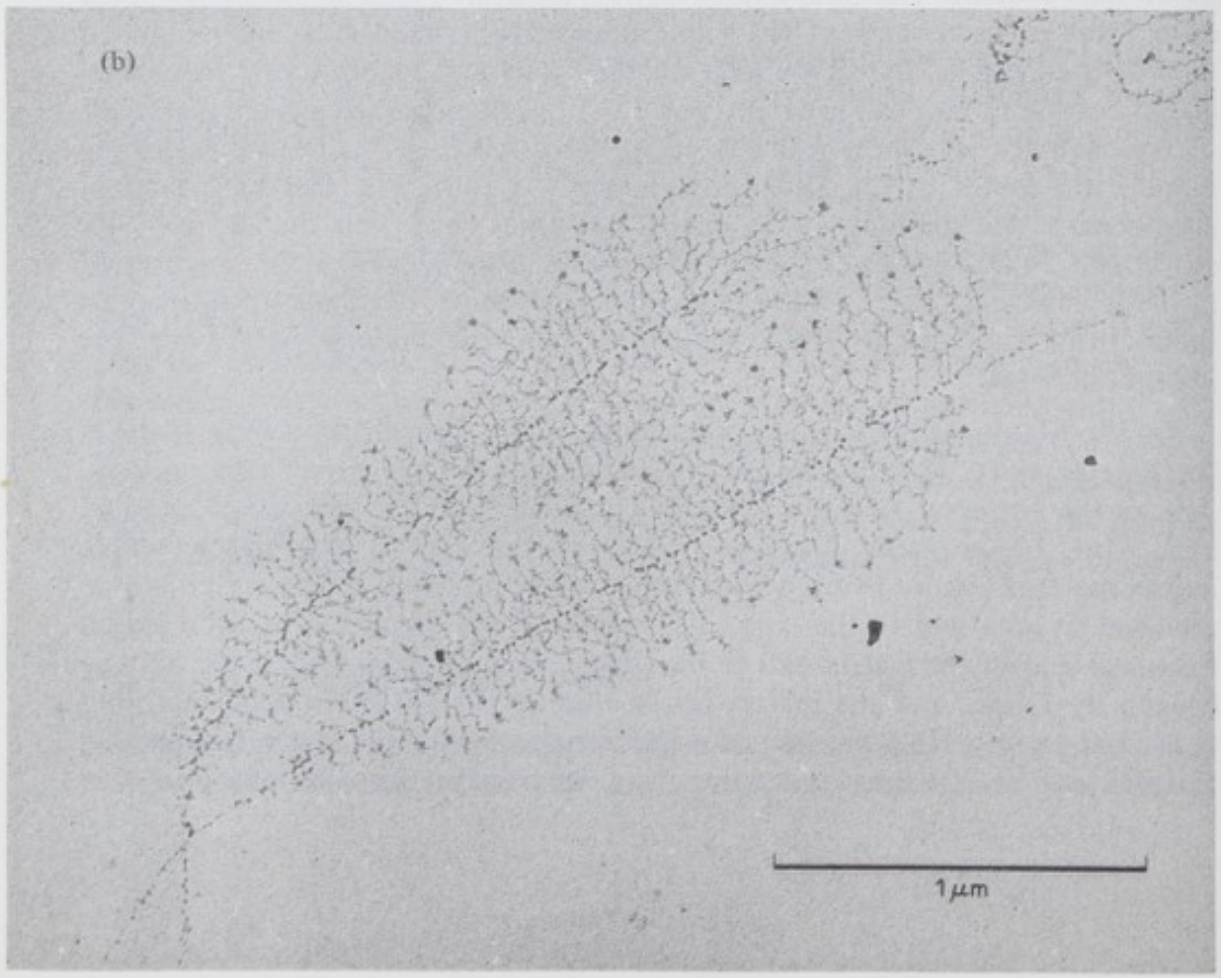

(b)
1μm

Figure 18.6 The visualisation of transcription.

(a) An electron micrograph of nucleolar genes isolated from an oocyte of the spotted newt, *Triturus viridescens*. Staining has revealed that the long axial fibres are a complex of DNA and protein and that the lateral fibrils are a complex of RNA and protein. Note that the axial DNA fibre consists of alternating transcriptionally active and inactive regions. (By courtesy of Oscar L. Miller and Barbara R. Beatty. Visualisation of nucleolar genes. *Science, N.Y.*, **164**, 955–7, 1969.)

(b) Two nucleolar gene complexes stretched during preparation. Each axial DNA fibre is being repeatedly transcribed from left to right so that there is a gradient of ribonucleoprotein fibril length from one end of the matrix to the other. At the base of each lateral ribonucleoprotein fibril is a densely staining granule believed to be a molecule of RNA polymerase. At each end of the gene complexes are the untranscribed 'spacer' regions. (By courtesy of O. L. Miller and B. A. Hamkalo. Visualisation of RNA synthesis in chromosomes. *Int. Rev. Cytol.*, **33**, 1–23, 1972.)

between each repeating unit there was a region of visually inactive *spacer* DNA that was not undergoing transcription.

Donald Brown and his colleagues have examined partially denatured rDNA from *Xenopus* and found a similar pattern of organisation; partial denaturation allows the strands in the weakly bonded A-T rich regions to separate while the more strongly bonded G-C rich regions remain double-stranded. These electron micrographs revealed a regularly repeating pattern of denatured loops (that is, A-T rich spacers) and double-stranded DNA (that is, G-C rich genes for ribosomal RNA); each repeat unit (figure 18.7a) consisted of two lengths of rDNA and two lengths of A-T rich DNA.

Figure 18.7b shows how the 28*S* and 18*S* genes and A-T rich regions are arranged along the molecule of DNA and how they are transcribed; this arrangement is repeated 450 times within each nucleolar organiser region and within each extra-chromosomal nucleolus. Each repeat unit is separated from the next by a non-transcribed spacer about 5000 nucleotide pairs long; the function of this spacer is unknown, but one possibility is that it could be a very large promoter region for binding the RNA polymerase molecules. Both the 18*S* and 28*S* genes are preceded by short lengths of A-T rich DNA (about 950 nucleotide pairs), and these, together with the 18*S* and 28*S* genes, are transcribed on to a single molecule of 40*S* precursor rRNA, later enzymatically cleaved into 18*S* and 28*S* rRNA and two small RNA fragments. The 18*S* and 28*S* rRNA eventually passes into the cytoplasm, but the two small RNA fragments, the transcripts of the two short A-T rich regions, remain within the nucleus; their function is unknown but they could be involved in the assembly of the ribosomes. Each 5*S* gene, also associated with a non-transcribed spacer about 900 nucleotide pairs long, is transcribed separately from the 18*S* and 28*S* rRNA genes. It is interesting to note that in the anucleolate mutant of *Xenopus* no 5*S* rRNA is synthesised, although all the 5*S* rRNA genes are apparently intact—thus the 5*S*, 18*S* and 28*S* rRNA genes are under coordinate control and so constitute the only established example of an operon in a higher organism.

Pre-mRNA

In microbial systems the immediate product of transcription is messenger RNA

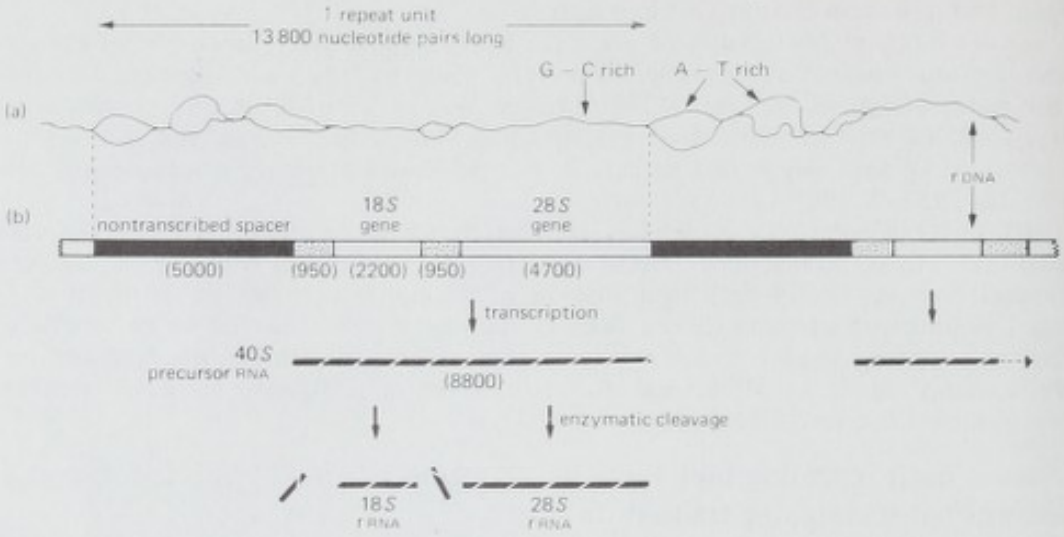


Figure 18.7 The ribosomal RNA genes in *Xenopus*. Partial denaturation of the ribosomal DNA (above) reveals that it is made up of many serially replicated units containing a regular array of G–C rich (double stranded) and A–T rich (denatured) regions. Within each nucleolar organiser region there are about 450 of these serially replicated units, each consisting of one 18*S* gene, one 28*S* gene, two short transcribed sequences of unknown function and a large non-transcribed spacer (centre). Each unit is transcribed to form a single molecule of 40*S* precursor RNA (below), later broken down to form a molecule of 18*S* rRNA, a molecule of 28*S* rRNA and two small RNA fragments. The figure shows the approximate numbers of nucleotide pairs (DNA) or nucleotides (RNA) in each nucleic acid segment.

(or sometimes rRNA or tRNA), but in eucaryotic systems these RNAs are first synthesised as very long molecules, which are subsequently cut up by nucleases into individual molecules of messenger RNA. When the loop of a lampbrush chromosome is transcribed the RNA transcripts are very long and probably represent the transcription of an entire loop containing a very large number of genes or gene copies, but such long RNA molecules are never found in the cytoplasm. In ducks the RNA isolated from erythroblast nuclei has an average size of 60*S* whereas the cytoplasmic messenger RNA is only 9*S*; furthermore, in erythroblasts there is probably only one copy per haploid chromosome set of each of the genes specifying the haemoglobin polypeptides. It appears that transcription produces a large precursor molecule (pre-mRNA) which is then broken down to form the much smaller molecules of cytoplasmic mRNA. Whereas most of the mRNA found in the cytoplasm is between 6 and 30*S* and has a relatively long life, most of the informational RNA in the nucleus is much larger, 60–90*S*, and relatively short-lived. After the breakdown of this informational RNA some of the smaller fragments, presumed to be mRNA, pass to the cytoplasm, but other fragments never leave the nucleus and have no identifiable function (see below).

Repetitive DNA

Although selective gene replication must give rise to repetitive DNA sequences, either as chromosomal or extra-chromosomal copies, repetitive DNA is found

not only after gene amplification but also as a normal component of the eucaryotic genome. Repetitive DNA is most easily detected by examining the *rate* at which single strands of DNA reanneal to reform double helical regions: the more times a given nucleotide sequence is present, the greater will be the chance that two complementary sequences will come together, and the more rapid will be the annealing of the single-stranded DNA. Sometimes these repetitive sequences have a higher or lower G-C content than the total DNA and so can be separated by centrifugation in a caesium chloride gradient; any DNA that can be separated in this way is called *satellite* DNA. Some DNA is highly repetitive and certain DNA sequences up to 300 nucleotide pairs long appear to be present in as many as 10^5 to 10^6 copies per genome. This very rapidly annealing DNA appears to be present in all eucaryotes; in the calf over 40 per cent of the DNA consists of sequences repeated 10^5 to 10^6 times, in mouse the corresponding figure is 10 per cent and in *Drosophila* about 6 per cent. The bulk of this highly repetitive DNA is located in heterochromatin, particularly in the regions of the centromeres, and its functions are unknown.

Other repetitive DNA, termed middle-repetitive DNA (MR-DNA) consists of sequences repeated 10 to 10^4 times per genome and made up of 100 to 150 nucleotide pairs. In *Drosophila* about 15 per cent of the DNA (1.8×10^7 nucleotide pairs) is MR-DNA and about 79 per cent (9.5×10^7 nucleotide pairs) consists of unique DNA that is presumed to be the structural genes. The MR-DNA is made up of sequences about 100 to 150 nucleotide pairs long and each sequence is repeated, on the average, about thirty to thirty-five times per genome; it is interspersed between sequences of unique DNA about 750 nucleotide pairs long.

It was first shown that eucaryotic DNA contains both unique and repetitive sequences of DNA in 1968, and since then an increasing amount of research has been directed to elucidating the functions of this repetitive DNA and to determining how these two fundamentally different types of DNA are organised into the chromomeres. However, the problem is not as simple as it appears as, even assuming that the unique DNA is the genes themselves, we still do not know just how many genes are present in each chromomere or band.

Chromomeres and Genes

The simplest notion is that each chromomere contains a single gene or gene complex and so is identified with a single gene product. An average chromomere may contain sufficient unique DNA to encode some thirty to thirty-five average sized polypeptides, so that if it contains only a single structural gene the remainder of this unique DNA is most probably associated with the specific but unidentified control system that must exist for regulating the activity of the associated structural gene (figure 18.8a). Another possibility is that each chromomere contains a number of copies of a single gene. We have already seen this situation in the nucleolar organiser regions (which can be regarded as giant chromomeres) where the structural genes for ribosomal RNA are laterally replicated and interspersed with lengths of DNA of unknown function

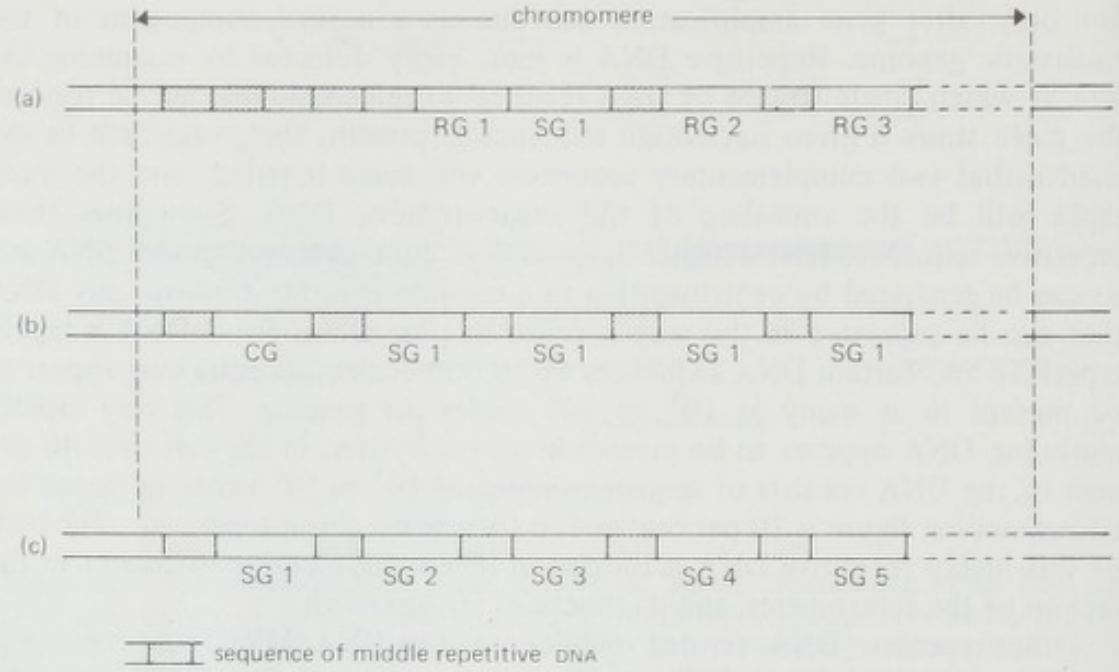


Figure 18.8 Chromomeres and genes. Three suggestions as to how genes and middle repetitive DNA might be organised within a chromomere.

(a) Each chromomere consists of a single structural gene (SG), a number of regulatory genes (RG) and some sequences of middle repetitive DNA.

(b) The chromomere consists of a serially replicated structural gene and one or more control genes, interspersed with sequences of middle repetitive DNA.

(c) The chromomere consists of many different structural genes interspersed with sequences of middle repetitive DNA.

(figure 18.7) and it is tempting to speculate that some chromomeres may likewise contain a number of identical copies of a gene, each separated by a piece of MR-DNA (figure 18.8b). However, the biochemical data make it unlikely that many chromomeres contain serially repeated genes, as reiterated sequences 1000 to 1500 nucleotide pairs long (the size of an average gene) have not been isolated from euchromatic DNA. A third possibility is that a chromomere contains many different, but probably related, genes. James Bonner and his associates (1973) have calculated that there are about 4500 different *types* of MR-DNA sequences in *Drosophila*, a figure remarkably close to the number of chromomeres; they also calculated that each chromomere contains enough unique DNA to encode some thirty average-sized protein molecules, a number which coincides with the number of copies per genome of each species of MR-DNA. On the basis of these coincidences James Bonner has suggested that within each chromomere there are thirty or so unique DNA sequences (that is, genes) separated from each other by identical or nearly identical sequences of MR-DNA, and that each chromomere contains a different species of MR-DNA—that is to say, each species of MR-DNA is chromomere specific (figure 18.8c).

It must be emphasised that models which envisage the chromomere as containing many different genes have been proposed largely to explain the

biochemical evidence obtained by the analysis of eucaryotic DNA; they are *not* in accord with the genetic evidence. The most compelling evidence against a chromomere containing many different genes is that no chromomere or band is known to have more than one primary activity; for example, different mutations, which affect the structure of a particular band, always influence the expression of the same character, and it is only in exceptional circumstances that they are able to complement one another in doubly heterozygous flies (that is, $a_1\ a_2^{+}/a_1^{*}\ a_2$). On the other hand, a few specific bands do show intra-band complementation, suggesting that these bands contain genetic elements that are functionally more complex than a single gene; nevertheless, the main weight of the evidence is in accord with the notion that most chromomeres contain a genetic unit comprising a single complementation group, or in other words a single gene.

Chromosomal RNA

There is only one clue as to a possible function of the transcripts of the MR-DNA sequences, which we know never leave the nucleus. In addition to DNA, proteins and pre-mRNA there is another type of RNA found associated with the chromosomes, the chromosomal RNA. This chromosomal RNA occurs in short lengths up to 400 nucleotides long, it has a heterogeneous but tissue-specific base composition and it is found bound to the DNA and histone protein in eucaryotic chromosomes. Further, this chromosomal RNA hybridises with the DNA contained in the MR-DNA fractions and it seems not unlikely that this RNA is the transcript of the MR-DNA that is released when the pre-mRNA is broken down. It has been suggested that these specific molecules of chromosomal RNA act as adaptor molecules which endow the non-specific histones with the specificity necessary for them to act as regulatory proteins.

Summary of Key Points

(1) The variations between different types of differentiated cell are due largely to differences in their protein compositions. Differentiation itself has been achieved by carefully programming differential gene activity, so that particular genes are activated and inactivated as differentiation and development proceed.

(2) Nuclear transplantation experiments and organ regeneration experiments show that differentiation is often a reversible process.

(3) Although embryonic cells are programmed to develop into a particular structure the programme can be erased, and reset so that the cells can now develop into a quite different structure.

(4) Differential gene activity can be directly visualised by the occurrence of puffing in the salivary gland chromosomes of Diptera.

(5) Among the mechanisms that can control the amounts and types of gene products present in a cell are:

(i) differential gene replication;
(ii) control of transcription by cytoplasmic repressors;
(iii) control of transcription by facultative heterochromatisation;
(iv) control of translation by varying the half life of mRNA and by only activating informational RNA when it is actually required as a template for protein synthesis.

(6) In the lampbrush chromosomes the genetic information *may* be serially replicated around the loops; the loops are actively engaged in transcription.

(7) In each nucleolar organiser region and in each nucleolus the ribosomal RNA genes of *Xenopus* are serially replicated 450 times; each pair of one 18*S* and one 28*S* gene is separated from the next pair by a length of non-transcribed spacer DNA.

(8) Eucaryotic chromosomes contain both unique DNA (sequences present only once per genome) and reiterated DNA (identical or similar sequences present many times per genome).

(9) It is not known how the reiterated and unique DNA is organised into the chromomeres, but the genetic evidence suggests that each chromomere contains just one structural gene.

(10) Chromosomal RNA is found bound to DNA and histone along the length of eucaryotic chromosomes; this chromosomal RNA may be a transcript of the middle repetitive DNA and it may act as adaptor molecules to endow the non-specific histones with the specificity necessary for them to act as specific repressor molecules.

(11) In many instances we know at which level the control of gene activity is taking place, but it is only in the controlling element systems in maize that the genetic elements involved in gene regulation have been positively identified.

(12) Special techniques described are starch gel electrophoresis and DNA/RNA hybridisation assay.

Bibliography

Section A: General Reading

The following textbooks and monographs are recommended to the student who wishes to consolidate and to amplify the information given in this book.

Beckwith, J. and Zipser, D. (1970). *The Lactose Operon*, Cold Spring Harbor Laboratories, New York.

An important collection of papers on the structure and function of the *lac* operon in *E. coli*.

Clarke, C. A. (1970). *Human Genetics and Medicine*, Arnold, London.

A short but knowledgeable account of the clinically important aspects of human genetics.

Drake, J. W. (1970). *The Molecular Basis of Mutation*, Holden-Day, San Francisco.

A detailed account of mutation and the mutation process, primarily with regard to bacteriophage systems.

Du Praw, E. J. (1970). *DNA and Chromosomes*, Holt, Rinehart and Winston, New York.

The molecular biology of chromosomes.

Fincham, J. R. S. and Day, P. R. (1971). *Fungal Genetics* (third edition), Blackwell, Oxford.

A specialised monograph on all aspects of fungal genetics.

Hartman, P. E. and Suskind, S. R. (1969). *Gene Action* (second edition), Prentice-Hall, Englewood Cliffs.

A lively account of how genes work, with an emphasis on experimentation.

Hayes, W. (1969). *The Genetics of Bacteria and their Viruses* (second edition), Blackwell, Oxford.

A detailed account embracing all aspects of the genetics of bacteria and bacteriophage.

Hayes, W. (ed.) (1973). *Advances in Molecular Biology, British Medical Bulletin*, **29**, No. 3.

Fifteen original articles reviewing the most recent advances in molecular genetics.

Lewin, B. M. (1974). *Gene Expression I. Bacterial Genomes,* Wiley, Chichester.

The most detailed account of the replication and function of the genetic material of bacteria.

Markert, C. L. and Ursprung, H. (1971). *Developmental Genetics,* Prentice-Hall, Englewood Cliffs.

A critical account of the role of the genes during cell differentiation.

Pontecorvo, G. (1958). *Trends in Genetic Analysis,* Oxford University Press, London.

A critical discussion of the analysis of genetic fine structure.

Stent, G. S. (1970). *Molecular Genetics,* Freeman, San Francisco.

An account that places particular emphasis on the interpretation of experimental results.

Sturtevant, A. H. (1965). *A History of Genetics,* Harper & Row, New York, Evanston and London.

A fascinating and often first-hand account of the development of genetics from Aristotle to the discovery of recombination in bacteriophage.

Swanson, C. P., Merz, T. and Young, W. J. (1967). *Cytogenetics,* Prentice-Hall, Englewood Cliffs.

A straightforward account of both classical and molecular cytology and cytogenetics.

Watson, J. D. (1968). *The Double Helix,* Weidenfeld and Nicolson, London.

A first-hand account of the discovery of the structure of DNA.

Watson, J. D. (1970). *Molecular Biology of the Gene* (second edition), Benjamin, New York.

A lively introduction to biochemical and molecular genetics.

Whitehouse, H. L. K. (1973). *Towards an Understanding of the Mechanism of Heredity* (third edition), Arnold, London.

A series of lucid and critical essays tracing the development of ideas about heredity from the earliest times to the present day. This book is particularly recommended to the more enquiring student, particularly the chapter on genetic recombination.

Section B: Collections of Reprints

The first three volumes are collections of articles reprinted from *Scientific American.* These easily understood articles contain a considerable amount of experimental detail and report new and exciting developments in particular fields of research. They are highly recommended.

Haynes, R. H. and Hanawalt, P. C. (eds.) (1968). *The Molecular Basis of Life,* Freeman, San Francisco.

Thirty-five articles describing much of the research referred to in this book.

Hanawalt, P. C. and Haynes, R. H. (eds.) (1973). *The Chemical Basis of Life,* Freeman, San Francisco.

Thirty-seven articles surveying recent developments in molecular and cell biology.

Srb. A. M., Owen, M.D. and Edgar, R. S. (eds.) (1970). *Facets of Genetics,* Freeman, San Francisco.

Thirty-five articles on aspects of genetics as diverse as the fine structure of the gene, differentiation, and evolution.

The remaining volumes are collections of reprints from scientific journals, and between them they contain many of the most important papers in the history of genetics.

Adelberg, E. A. (ed.) (1960). *Papers on Bacterial Genetics,* Little, Brown, Boston.

Twenty-seven original research articles describing much of the early work on bacterial genetics.

Peters, J. A. (ed.) (1959). *Classic Papers in Genetics,* Prentice-Hall, Englewood Cliffs.

A collection of very important papers up to 1955, including a translation of Mendel's paper describing his experiments with peas; there is a short critical appraisal of each paper.

Stent, G. S. (ed.) (1960). *Papers on Bacterial Viruses,* Little, Brown, Boston.

Twenty-five research papers describing the discovery of bacteriophage, the structure of DNA, lysogeny, and transduction.

Taylor, J. H. (ed.) (1965). *Selected Papers on Molecular Genetics,* Academic Press, New York and London.

An important collection of papers on biochemical genetics, the nature and replication of the genetic material, genetic recombination and gene function.

Section C: Original Research Papers

To read and understand a scientific paper is often much more difficult than to write one. The following papers are recommended both for their clarity of

presentation and for their scientific merit; they show very clearly how many great scientific discoveries depend not so much on elaborate experimentation but rather on critical reasoning followed by carefully designed but relatively simple experiments.

Ames, B. N., Durston, W. E., Yamasaki, E. and Lee, F. D. (1973). Carcinogens are mutagens; a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. U.S.A.*, **70**, 2281-5.

Mutagens recognise specific nucleotide sequences in DNA.

Beadle, G. W. and Tatum, E. L. (1941). Genetic control of biochemical reactions in *Neurospora*. *Proc. Nat. Acad. Sci. U.S.A.*, **27**, 499-506.

The one gene-one enzyme hypothesis; reprinted in Taylor (1965).

Benzer, S. (1955). Fine structure of a genetic region in bacteriophage. *Proc. Nat. Acad. Sci. U.S.A.*, **41**, 344-54.

Fine-structure analysis of the T4 *r*II region; reprinted in Peters (1959) and Stent (1960).

Brenner, S., Jacob, F. and Meselson M. (1961). An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature, Lond.*, **190**, 576-81.

The discovery of mRNA; reprinted in Taylor (1965).

Brenner, S., Stretton, A. O. W. and Kaplan, S. (1965). Genetic code; 'nonsense' triplets for chain termination and their suppression. *Nature, Lond.*, **206**, 994-8.

The decoding of nonsense; simple experiments and carefully argued reasoning.

Cairns, J. (1963). The bacterial chromosome and its manner of replication as seen by autoradiography. *J. Molec. Biol.*, **6**, 208-13.

Visualisation of the *E. coli* chromosome.

Crick, F. H. C., Barnett, L., Brenner, S. and Watts-Tobin, R. J. (1961). General nature of the genetic code for proteins. *Nature, Lond.*, **192**, 1227-32.

The code is a commaless, nonoverlapping triplet code; reprinted in Taylor (1965).

Delbrück, M. and Bailey, W. T. (1947). Induced mutations in bacterial viruses. *Cold Spring Harb. Symp. quant. Biol.*, **11**, 33-7.

First demonstration of induced mutation in phage; reprinted in Taylor (1965).

Demerec, M. and Demerec, Z. E. (1956). Analysis of linkage relationships in *Salmonella* by transduction techniques. *Brookhaven Symp. Biol.*, **8**, 75-87.

One of the many fine papers on the analysis of genetic fine structure in *Salmonella.*

Goulian, M. (1969). Synthesis of viral DNA. *Sci. J.*, March, 35-42.

The *in vitro* synthesis of infective ϕX174 DNA.

Huberman, J. A. and Riggs, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J. Molec. Biol.*, **32**, 327-41.

Autoradiography of mammalian DNA.

Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Molec. Biol.*, **3**, 318-56.

Development and proof of the operon hypothesis; reprinted in Taylor (1965).

Lederberg, J. (1974). Gene recombination and linked segregations in *Escherichia coli. Genetics*, **32**, 505-25.

Early experiments on bacterial conjugation; reprinted in Adelberg (1960) and Peters (1959).

Mendel, G. J. (1866). Experiments in plant hybridisation. Translated in *J. R. Hort. Soc.* (1901), **26**, 1-32.

The birth of the science of genetics—a wonderful example of a brilliant analytical mind at work; reprinted in Peters (1959).

Meselson, M. and Stahl, F. W. (1958). The replication of DNA in *Escherichia coli. Proc. Nat. Acad. Sci. U.S.A.*, **44**, 671-82.

Classic experiment demonstrating the semi-conservative replication of DNA; reprinted in Taylor (1965).

Miller, O. L. (jr.), Beatty, B. R., Hamkalo, B. A. and Thomas, C. A. (jr.) (1970). Electron microscopic visualization of transcription. *Cold Spring Harb. Symp. quant. Biol.*, **35**, 505–12.

The direct visualisation of genes in action.

Muller, H. J. (1927). Artificial transmutation of the gene. *Science, N.Y.*, **66**, 84-7.

The first demonstration of induced mutation; reprinted in Peters (1959).

Speyer, J. F., Lengyel, P., Basilio, C. and Ochoa, S. (1962). Synthetic polynucleotides and the amino-acid code, IV. *Proc. Nat. Acad. Sci. U.S.A.*, **48**, 441-8.

Triplet assignments using random polynucleotides; reprinted in Taylor (1965).

Sutton, W. S. (1903). The chromosomes in heredity. *Biological Bulletin (Woods Hole)*, **4**, 24–39.

The exposition of the chromosome theory of heredity; reprinted in Peters (1959).

Watson, J. D. and Crick, F. H. C. (1953). Molecular structure of nucleic acids: a structure of deoxyribose nucleic acid. *Nature, Lond.*, **171**, 737–8.

The elucidation of the structure of DNA; reprinted in Peters (1959) and Taylor (1965).

Watson, J. D. and Crick, F. H. C. (1954). The structure of DNA. *Cold Spring Harb. Symp. quant. Biol.*, **18**, 123–31.

The implications of the Watson–Crick model; reprinted in Taylor (1965).

Wollman, E. L., Jacob, F. and Hayes, W. (1956). Conjugation and genetic recombination in *Escherichia coli* K12. *Cold Spring Harb. Symp. quant. Biol.*, **21**, 141–62.

Genetic analysis using Hfr strains; reprinted in Adelberg (1960).

Yanofsky, C., Carlton, B. C., Guest, J. R., Helinski, D. R. and Henning, O. (1963). On the colinearity of gene structure and protein structure. *Proc. Nat. Acad. Sci. U.S.A.*, **51**, 266–72.

A demonstration of co-linearity in the *E. coli* tryptophan synthetase A protein system.

Glossary

The numbers refer to the pages where the definitions are more fully explained. The explanation of technical terms not listed in this glossary can be found in the text by reference to the index.

Adenosine triphosphate (ATP) A compound with high-energy phosphate bonds that provides the energy for many cellular processes. (237)

Aleurone The outermost and sometimes pigmented layer of the triploid endosperm. (66)

Amino acid One of a group of compounds with an amino ($-NH_2$) and a carboxyl (-COOH) group. The twenty common α-amino acids are the building blocks of proteins. (5, 6, 179)

Amino-acid substitution The replacement of one amino acid by another at a particular position within a polypeptide because of an alteration in the genetic information. (188, 209)

Aminoacyl synthetases The enzymes which activate amino acids by reaction with ATP and which couple these activated amino acids to their respective tRNA molecules; there is at least one aminoacyl synthetase for each amino acid. (237)

Amplification An increase in the dose of a specific gene by making a number of copies on extra-chromosomal DNA. Amplification is *not* used to describe the serial replication of a gene along a chromosome. (325)

Anticodon The sequence of three nucleotides in a molecule of tRNA that recognises a complementary sequence of nucleotides (codon) on a molecule of messenger RNA. (206, 236)

Alleles Alternative forms of the same gene. (3)

Autoradiograph A 'photograph' produced by labelling a structure such as a bacterial chromosome with a radioactive label and overlaying with a photographic emulsion. The emissions from the radioactive label expose the emulsion and so reveal the size and shape of the underlying structure. (31)

Autosomes Any chromosome that is not directly involved in the determination of sex. (86)

Auxotroph A mutant micro-organism that will only grow when a particular growth factor is supplied. (105)

Back-cross A cross between an organism and one of its parents. (67)

Bacteriophage (phage) A virus that infects and replicates in bacteria. (35)

Base analogue A compound that may sometimes replace one of the naturally occurring nucleotide bases. (170)

Base substitution The replacement of one base (or base pair) in a nucleic acid by a different base (or base pair). (155)

Carcinogen An agent that causes cancer. (303)

Catabolite repression The decreased rate of synthesis of enzymes involved in

the metabolism of glucose when bacteria are grown on a glucose-rich medium. (270)

Centromere A specialised region of a chromosome to which the spindle fibres attach at cell division. (49)

Chain-termination triplet A nonsense codon. (157, 213)

Chiasma The cross-like structure generated during recombination at the point where non-sister chromatids appear to break and exchange genetic material. (54, 273)

Chromatid One of the two identical daughter strands produced when a chromosome replicates; once the centromeres separate each chromatid becomes a chromosome. (49)

Chromatin The nucleo-histone complexes making the chromosomes. (48)

Chromomere A laterally differentiated region of a chromosome. In meiotic chromosomes the chromomeres appear like beads on a string, but in the giant salivary gland chromosomes each chromomere is seen as a transverse band. (53, 61)

Chromosome Structures carrying a linear sequence of genetic information. In procaryotes it is a molecule of naked DNA, in eucaryotes it is a nucleo-protein complex visible under the light microscope. (2, 48)

Circularly permuted DNA. The population of linear molecules of DNA produced as if by breaking open circular molecules at random points. (37)

Cis arrangement A double heterozygote in the coupling phase (*AB*/*ab*); usually used for closely linked mutant sites. (262)

Clone A group of cells all descended from a single ancestral cell. (162)

Codon The sequence of three nucleotides in DNA or RNA that codes for a particular amino acid or for the termination of polypeptide synthesis. (8, 195)

Cohesive ends Single-stranded and base-complementary sequences of nucleotides at opposite ends of a molecule of DNA. (40)

Co-linearity The concept that the linear sequence of nucleotides in DNA directly determines the linear sequence of amino acids in a protein. (191)

Colony A group of continguous bacteria growing on the surface of a solid medium; usually they are all descended from the same ancestral bacterium. (14)

Complementation The ability of two recessive mutants to make good each other's defect when they are present in the same cell but on different chromosomes. (113, 186)

Concatenate A newly synthesised molecule of DNA of greater than unit length. (37)

Controlling element Genetic units in maize which regulate the activity of other genes and which can transpose from one location in the genome to another. (308)

Conjugation In bacteria, the coming together of a donor and a recipient cell and the formation of a cytoplasmic bridge between them. (122)

Conditional lethal mutants Mutants which are only viable under one particular set of defined growth conditions. (139)

Corepressor A low molecular weight compound that binds to a gene-specified repressor protein to form an active repressor complex. (257)

Coupling Two pairs of linked genes are in the coupling phase when both dominant alleles were contributed by one parent and both recessive alleles by the other parent (that is, *AB*/*ab*). See *repulsion, cis, trans.* (75)

Crossing-over The process of genetic recombination that gives rise to new combinations of linked genes. (54, 73)

Degeneracy The genetic code is degenerate because most amino acids are encoded by two or more codons. (195)

Deletion The loss of one or more adjacent nucleotide pairs from a chromosome. (119, 154)

Density-gradient centrifugation A sensitive technique for separating related molecules which have slightly different densities. (35)

Diploid An individual or cell with two homologous sets of chromosomes, one derived from each parent. (4)

DNA-*dependent* DNA *polymerase* An enzyme that makes new DNA against a DNA template. (28)

DNA-*dependent* RNA *polymerase* An enzyme that makes RNA against a DNA template (transcription). (220)

Dominant gene Usually describes the gene which is expressed in a heterozygote, but which more correctly describes the *character* that is expressed in the F_1 when two pure-breeding strains differing only by this character are crossed. (3, 188)

Endonuclease An enzyme that makes breaks in the sugar-phosphate backbone of a molecule of nucleic acid. (44)

Enzymes Protein molecules or molecular complexes that catalyse biochemical reactions. (178)

Episome An additional genetic element that can exist either as an autonomous entity or inserted into the continuity of the chromosome of a host cell. (124)

Eucaryote An organism with cells that possess nuclear membranes and certain organelles such as mitochondria. (30)

Euchromatin The parts of a eucaryotic chromosome that show the normal cycle of condensation at cell division. (49)

Exonuclease An enzyme that digests a molecule of nucleic acid from one end. (39)

Factors Small protein molecules that assist in the regulation of transcription and translation. (225, 243)

Feedback inhibition Occurs when the end-product of a biochemical pathway inhibits the *activity* of the first enzyme in the pathway. (252)

F-factor The fertility factor of *Escherichia coli.* (123)

F-prime factor (F') An F-factor carrying certain bacterial genes. (132)

Frameshift mutation A mutation which adds or deletes a base pair from DNA so that the genetic code is read out of phase. (157)

Gene The genetic unit of function; it may specify a polypeptide, a molecule of RNA, or it may play a role in the regulation of gene activity. (2)

Gene conversion Usually used to describe the process of non-reciprocal recombination that gives rise to aberrant segregations in tetrads. (277)

Genome The total gene content of a cell or organism. (4)

Genotype A description of the genetic constitution of a cell or organism. (3)

Haploid An organism or cell having one set of chromosomes. (4)

Haemoglobin A protein, found in the red blood cells, that carries oxygen around the body. (188)

Heterochromatin Chromatin that is relatively over or under-condensed at cell division; it is thought to be transcriptionally inactive. (284)

Heteroduplex A molecule of double-stranded DNA where the two strands do not have exactly complementary base sequences.

Heterozygote A cell or organism carrying different alleles of a gene on a pair of homologous chromosomes (for example, *Aa*). (3)

Homologous chromosomes A pair of similar chromosomes, one of paternal and one of maternal origin. They carry corresponding sequences of genes and pair together at meiosis. (3)

Homozygote A cell or organism carrying identical alleles of a gene on a pair of homologous chromosomes (for example, *AA* or *aa*) (3)

Inducer A low molecular weight molecule that binds to and inactivates a repressor, so causing the switching-on of gene activity. (9, 257)

Inducible enzyme An enzyme that is produced at an increased rate when a specific inducer molecule is present. (9, 257)

Linkage Genes on the same chromosome are said to be linked as they tend to be inherited together. (72)

Linkage map The order of mutant sites or genes along a chromosome, as deduced from recombination data. (77)

Locus The location of a gene on a chromosome. (72)

Lysogenic bacterium A bacterium carrying a prophage. (143)

Meiosis The process of cell division, which halves the number of chromosomes in a diploid organism so as to form haploid gametes or spores. (4, 53)

Merozygote A partially diploid zygote as is produced in bacterial conjugation and transformation. (126)

Messenger RNA A molecule of RNA that acts as a template for translation and which is a transcript of chromosomal DNA. (6, 218)

Missense mutation A mutation that changes a codon for an amino acid into a codon for a different amino acid. (157)

Mitosis The process of normal cell division. (4, 49)

Multiplicity of infection (M.O.I.) The ratio of phage to bacteria in an infection. (109)

Mutagen An agent that induces mutation. (168)

Mutant An organism carrying a specific mutation and displaying an altered phenotype. (3)

Mutation A sudden inheritable change in the genotype of an organism. (3, 153)

Negative control Biological activity only occurs in the *absence* of a specific repressor molecule. (268)

Nonsense codon A sequence of three nucleotides on a molecule of mRNA that signals the termination of transcription. (157, 213)

Nucleolar organiser A region on a eucaryotic chromosome that is associated with the formation of a nucleolus. It contains genes that specify ribosomal RNA. (49)

Nucleolus A granular structure found in eucaryotic cells attached to a specific chromosomal site; it is the site of ribosomal RNA synthesis. (2)

Nucleoside One of four nitrogenous bases linked to a molecule of ribose or deoxyribose. (18)

Nucleotide A nucleoside with an attached phosphate group. (18)

Operator A region or gene to which a molecule of repressor binds so as to regulate the activity of closely linked structural genes. (8, 257)

Operon A group of structural genes under coordinate control and the operator and promoter genes that control them. (8, 257)

Pericarp A layer of maternal tissue covering a seed. (66)

Phenotype The appearance or observable properties of an organism. (3)

Photoreactivation The repair of u.v.-damaged DNA by the cleaving apart of pyrimidine dimers. (292)

Polar mutation A nonsense mutation in a gene, which results in the decreased activity of other more distal genes in the same operon. (248)

Polypeptide A compound made up of two or more amino acids joined together by peptide bonds. (6, 178)

Polyribosome (polysome) A complex of mRNA and ribosomes actively engaged in protein synthesis. (232)

Position effect The modification of the expression of a gene by moving it from one position in the genome to another. (306)

Positive control Biological activity only occurs in the *presence* of a gene-specified regulatory protein. (268)

Procaryotes Organisms lacking a nuclear membrane and certain organelles such as mitochrondria. (30)

Promoter A region on a molecule of DNA where RNA polymerase binds so as to initiate transcription. (225, 265)

Prophage, A phage chromosome that has been integrated into the continuity of the bacterial chromosome. (143)

Protein A molecular complex made up of one or more folded and superfolded polypeptide chains. (5, 6, 178)

Prototroph A wild-type micro-organism that can grow on minimal medium. (105)

Pyrimidine dimer The main effect of u.v. irradiation on DNA is to fuse adjacent pyrimidines into a dimer. (292)

Recessive gene A gene that is expressed only when homozygous (that is *aa*). (3)

Regulator gene A gene specifying a specific repressor protein. (8, 257)

Repressible enzyme An enzyme whose rate of synthesis is decreased in the presence of certain metabolites. (255)

Repressor The protein product of a regulator gene that can combine with either the operator gene or with an inducer. (8, 257)

Repulsion Two pairs of linked genes are in the repulsion phase when the paternal and maternal chromosomes each carry one dominant and one recessive gene (that is *Ab/aB*). See *coupling, cis, trans.* (75)

Ribosomes Complexes of RNA and protein, the sites of protein synthesis. (2, 217)

RNA-*dependent* DNA *polymerase* An enzyme that synthesises DNA against an RNA template; reverse transcriptase. (227)

RNA-*dependent* RNA *polymerase* An enzyme that synthesises RNA against an RNA template; RNA replicase, RNA synthetase. (227)

Screening A technique to increase the proportion of a rare phenotype in a population so making its recovery easier. (162)

Selection technique A method permitting the recovery of organisms of a particular rare phenotype and the exclusion of the other much more common phenotypes. (105)

Semi-conservative replication The method whereby DNA replicates so as to produce two daughter molecules each consisting of one new and one parental strand. (24)

Sex chromosome Chromosomes actively concerned with the determination of sex; the X and/or Y chromosomes. (86)

Sex linkage Genes on the sex chromosomes do not assort independently of sex and show sex linkage. (88)

Site The position of a mutation within a gene. (113)

Structural gene A gene encoding a polypeptide, or a molecule of transfer or ribosomal RNA. (8, 257)

Temperate phage A phage that, when it infects a cell, may either establish itself as prophage or may replicate and destroy the host bacterium. (143)

Template A macromolecule that serves as a biological mould for the synthesis of a similar macromolecule. (24)

Terminal redundancy A terminally redundant molecule of DNA has the sequence of bases at one end of the molecule repeated at the other end. (37)

Testcross A cross designed to yield specific information on the linkage relationships betwen the genes involved. (67)

Tetrad A bivalent made up of paired homologous chromosomes at the first meiotic division; the four products of a single meiosis. (56, 92)

Trans arrangement A double heterozygote in the repulsion phase (*Ab/aB*); usually used for closely linked mutant sites. (262)

Transcription The transfer of the genetic information from a double-stranded molecule of DNA to a single-stranded molecule of RNA. (6)

Transdetermination The reprogramming of an apparently undifferentiated cell so as to alter its subsequent pattern of differentiation and development. (323)

Transduction The transferring of genes from one bacterial strain to another by a bacteriophage. (109)

Transfer RNA Adaptor molecules of RNA which can combine with a specific amino acid and with a specific codon on a molecule of mRNA. (8, 233)

Transformation The transfer of genetic information from one bacterium to

another by 'infecting' a recipient strain with purified DNA extracted from a donor strain. (14)

Transition A base-substitution mutation where a pyrimidine has replaced a pyrimidine and/or a purine has replaced a purine. (172)

Translation The synthesis of a polypeptide against an mRNA template. (8, 230)

Transversion A base substitution mutation where a pyrimidine has replaced a purine and/or vice versa. (172)

Triplet A codon (195)

Virulent phage A phage that must destroy a bacterial host cell upon infection. (35)

Virus A minute infectious particle only able to reproduce inside a living cell. (16)

Wild type The normal condition of an organism or gene; the reference type. (3)

Wobble The concept whereby a base in the third position of an anticodon can pair with any one of two or more bases in a codon. (246)

Zygote The diploid cell formed by the union of the male and female gametes. (4)

Author Index

Organism Index

1. Viruses

(a) Plant and Animal Viruses

(b) The RNA-containing Bacteriophages

(c) The DNA-containing Bacteriophages

2. Bacteria

3. The Plant Kingdom

(a) Fungi–Ascomycetes

(b) Algae

(c) Angiosperms

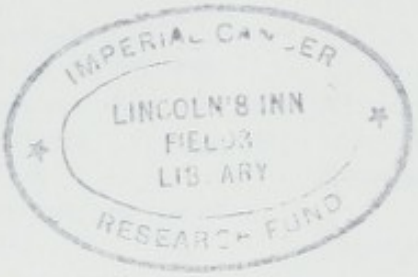
4. Animal Kingdom

(a) Protozoa

(b) Nematoda

(c) Arthropoda–Insecta

Subject Index



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