Recent Advances in HISTOPATHOLOGY

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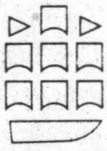

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Preface

Histopathology advances steadily and dramatic or — dare we admit? — exciting discoveries are few and far between. Some feel concerned about this and ponder over loss of prestige or even predict an inevitable decline. We do not agree. Histopathology has grown like the noble oak, gradually increasing the girth of its trunk, sending out more and more branches and sprouting fresh leaves each season. Our roots are firm in clinical medicine and in the basic sciences and, in turn, we continue to benefit both.

This volume, once again, presents a choice of topics ranging from the scientific to the clinical. As in previous volumes, we have sought broad reviews of reasonably well established observations. We are most grateful to all our contributors for their excellent products which, we hope, will be of interest to the reader.

April 1989

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Roddy MacSween, Glasgow

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Contents

1. DNA technology in histopathology

R. B. Goudie

The development of recombinant DNA technology ('genetic engineering') in the past 15 years has profoundly influenced our knowledge and understanding of how the body works in health and how some of its functions are disturbed in certain diseases. In medical practice its most important diagnostic applications have been in clinical genetics but it is now beginning to have a useful place in histopathological diagnosis. It has provided, for example, a virtually foolproof method of positively identifying tumours of T-lymphocytes and of reliably distinguishing them from T cell hyperplasias and other lymphoproliferative disorders.

In essence, recombinant DNA technology has made it possible to isolate and purify the 50 000 or so genes which human beings inherit and to obtain each in large quantities by in vitro culture techniques known as gene cloning. When a gene can be cloned it becomes possible to determine the sequence of bases — the chemical code in which genetic instructions are written — in the DNA molecule and to compare this with the base sequences of variant forms of the same gene which have arisen by mutation and may be the cause of disease. Purified gene preparations also permit large scale in vitro synthesis of the corresponding polypeptides, too little of which may be present in the tissues to extract and purify for adequate investigation of their structure and function. Any given cell only contains some 15 000 of the 50 000 possible kinds of polypeptide, and cloned DNA can be used to determine which particular genes are active, a valuable indication of growth, differentiation and other cellular activities of importance in histopathology. It should, however, be emphasised that the exploration of the whole human genome and its polypeptide products is still at an early stage and will certainly continue into the next century. Many of the genes discovered will be of little clinical importance but we can be sure that others will be found which will change the face of medicine.

This chapter provides a simplified account of the general principles of recombinant DNA technology and describes some of the ways in which it has already been used in histopathological investigations. First, a reminder of the normal structure and function of DNA might be helpful. For further information the reader is referred to Alberts et al (1983) and Lewin (1987).

DNA STRUCTURE

A diagram of part of a DNA molecule is shown in Figure 1.1. The vertical lines represent chains of alternating sugar (deoxyribose) and phosphoric acid units which form the so-called 'backbones' of the molecule. The form of the molecule with two backbones as shown in Figure 1.1(b) is called double stranded (ds) DNA. Note that the backbones are separated by paired organic bases adenine (A) and thymine

(T) or guanine (G) and cytosine (C), the whole structure resembling a ladder in which the base pairs are the steps and the uprights are the backbones of the molecule. In double stranded DNA the ladder is actually twisted in its long axis so that the uprights form the spiral structure of a double helix but this point is of little consequence in recombinant DNA technology. The following details are however essential for a proper understanding of the subject.

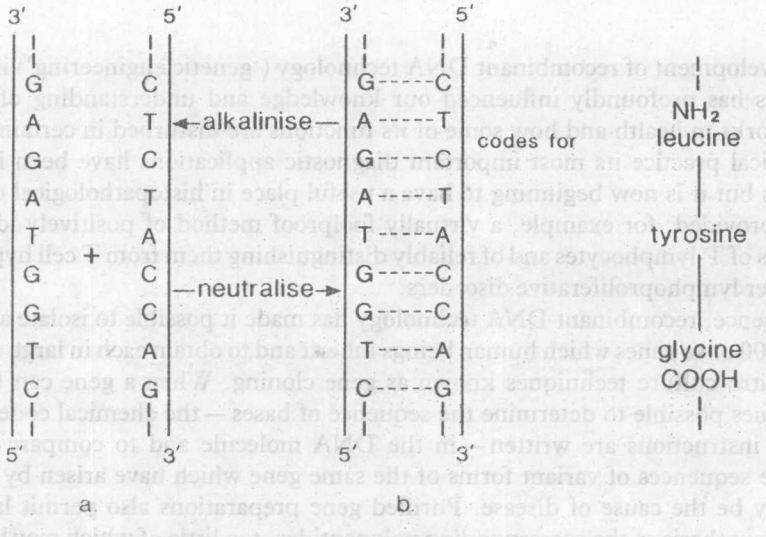

Fig. 1.1(a) Two single stranded (ss) DNA molecules formed by alkaline denaturation of the double stranded molecule shown in (b). On neutralising, the complementary ss DNA molecules reassociate (hybridise) to form the original double stranded structure. For an explanation of 5′ and 3′ see text. **(b)** Part of a double stranded (ds) DNA molecule showing a ladder-like structure. The uprights are the backbones of the molecule and consist of alternating deoxyribose and phosphoric acid units (not shown) while the steps consist of the complementary base pairs AT or GC. Part of the polypeptide chain coded by the DNA molecule in (b) is shown on the right.

Base pairing

1. In double stranded DNA the bases A and T are always paired together as are G and C. Each pair (AT or GC) is called a complementary base pair.
2. If double stranded DNA is denatured (e.g. by raising the pH of the solution) the complementary bases separate to form two single stranded (ss) DNA molecules (Fig. 1.1(a)). In appropriate conditions (e.g. on neutralisation) these will reassociate or hybridise again exactly as before in order to re-establish the mandatory pairing of the complementary bases.
3. Single stranded DNAs which can hybridise with each other to form a double stranded molecule must be complementary to each other. At least 17 consecutive complementary base pairs are required to form a stable union and the longer the complementary sequence the more stable the union that is formed.
4. A piece of single stranded DNA incorporating a radioactive label (e.g. ^{32}P in the phosphoric acid of its backbone) can therefore be used as a DNA 'probe' to detect and locate a complementary sequence in an 'unknown' sample of DNA. Oligo-

nucleotide probes about 20 bases long unite only with sequences which are exactly complementary, change in a single base of the target sequence preventing hybridisation. Probes which are hundreds of bases in length can hybridise with stretches of DNA which may not be entirely complementary.

The genetic code

DNA carries the inherited instructions for the sequence in which the various aminoacids must be linked together in order to form particular polypeptide chains. The instructions are written in a four lettered alphabet ATGC which is used to make a set of three lettered code names for the various aminoacids.

For example in Figure 1.1(b), reading down the strand on the right side of the molecule, the base triplet CTC codes for the aminoacid leucine, TAC for tyrosine, CAG for glycine and so on, the aminoacids of the corresponding polypeptide being linked together in that order.

Assuming that the piece of double stranded DNA shown in Figure 1.1(b) is part of a gene specifying the amino acids leucine-tyrosine-glycine, it is clear that in this example the base sequence coding for the polypeptide is present only in one strand, and the other strand represents a template on which complementary copies can be made. In order to obtain the aminoacid sequence leucine-tyrosine-glycine it is also essential that the correct 'reading frame' is used. If the C at the top of the right hand strand in Figure 1.1(b) is not read then the instruction becomes TCT (serine), ACC (threonine) etc. Finally from inspection of Figure 1.1(b) it is also evident that the genetic instructions must be read in the correct (5′ to 3′) direction. Read from below upwards, the DNA in our example would code for asparagine-histidine-leucine (GAC, CAT, CTC).

The principles of the genetic code as outlined above have applications in recombinant DNA technology. If, for example, enough of an interesting polypeptide can be isolated and a sequence of six or more aminoacids determined, it may then be possible to work back and synthesise a DNA probe and use it to isolate the whole gene.

FROM DNA TO POLYPEPTIDE

Although the base triplets on DNA encode the inherited instructions for the order in which the various aminoacids are arranged in the corresponding polypeptide, DNA is only indirectly involved in polypeptide synthesis. Figure 1.2 traces the process with the example of DNA illustrated in Figure 1.1 and shows the two major steps, the copying of the genetic instructions from DNA on to a complementary messenger RNA molecule — a process called transcription — and the translation of the message encoded in the messenger RNA molecule into the language of the polypeptides — the aminoacid sequence.

Messenger (m) RNA is a nucleic acid which differs from DNA in three important respects. (1) Its backbone contains *r*ibose (hence the name *R*NA) instead of *d*eoxyribose (as in *D*NA), (2) mRNA is always single stranded and (3) the base uracil (U) replaces thymine (T) of DNA (Fig. 1.2).

Synthesis of mRNA is catalysed by the enzyme DNA-dependent RNA polymerase (RNA polymerase for short) and uses the appropriate DNA sequence as a template.

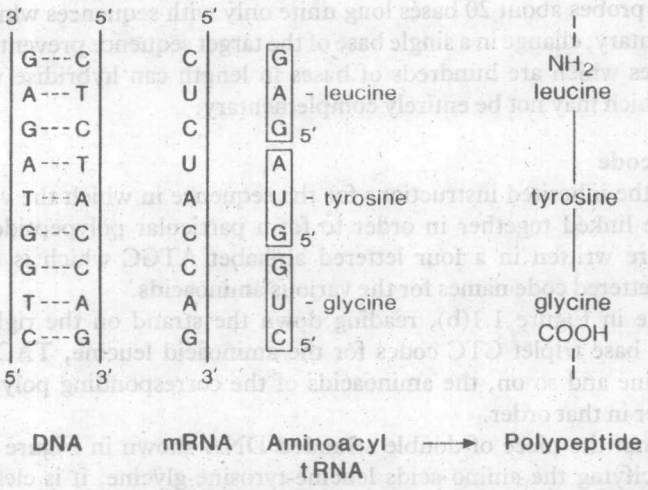

Fig. 1.2 Transcription of DNA to mRNA by complementary base pairing with the DNA strand on the left, and translation of mRNA to polypeptide with the help of tRNA.

The resulting mRNA transcript is complementary to the DNA strand on which it is formed (the left in the example in Figures 1 and 2). During synthesis the mRNA molecule always elongates in the 5′ to 3′ direction by the stepwise addition of complementary bases to its 3′ end as the RNA polymerase moves along the DNA strand which acts as the template.[1] (The symbols 3′ and 5′ actually indicate the sites of attachment of the chemical linkages between neighbouring ribose units in mRNA and deoxyribose units in DNA).

Complementary DNA and mRNA can be made to hybridise in the laboratory and so DNA probes can be made to detect mRNA and vice versa. Also 'sense' and 'antisense' mRNA molecules can hybridise.

Polypeptide synthesis takes place on the ribosomes. The order in which the aminoacids are added during synthesis of the growing polypeptide chain is determined by complementary base pairing between mRNA and the 'anticodons' of the various forms of transfer (t) RNA each of which is specific for a particular aminoacid (Fig. 1.2).

DNA SYNTHESIS

A detailed knowledge of DNA synthesis is required by those involved in making new DNA probes but is unnecessary for the purposes of this chapter. Suffice it to say that double stranded DNA first separates into two single strands each of which acts as a template for the formation of new strands of complementary bases which are then linked together by the enzyme DNA dependent DNA polymerase (DNA polymerase for short) and two exact copies of the original molecule are so produced. Synthesis can commence only where the template strand is hybridised to a complemen-

[1] To avoid ambiguity it is customary to describe the base triplets of the genetic code in terms of the bases present in mRNA written 5′ to 3′ from left to right. In reporting the base sequence of double stranded DNA the findings are given 5′ to 3′ for the strand which is *not* transcribed. Thus in Figure 1.2 the codons for leucine, tyrosine and glycine would be CUC, UAC and CAG respectively and the DNA coding for the sequence would be given as 5′ CTC, TAC, CAG 3′.

tary DNA strand which functions as a primer for DNA polymerase, and growth of the new strand always takes place in the 5′ to 3′ direction, points of fundamental importance in the polymerase chain reaction to be described later.

An alternative method found in cells infected with retro-viruses involves the use of RNA as a template for DNA synthesis. This process is catalysed by the enzyme RNA dependent DNA polymerase which in effect functions as a *reverse transcriptase* and has been of great value in the identification of genes by making it possible to obtain complementary DNA copies (*cDNA*) from mRNA. A great deal is known about the globin genes, for example, largely as a result of cDNA probes prepared from the mRNA present in reticulocytes.

OTHER ASPECTS OF DNA AND mRNA

The human genome is stored in 46 chromosomes (23 from each parent). Each chromosome contains a single tightly coiled thread-like DNA molecule forming a double helix of the order of 10^8 base pairs in length. If unravelled, such molecules would each be about two centimetres in length.

DNA molecules also exist which are much smaller than those just described and some of these have an important place in recombinant DNA technology. Most notable are the *plasmids*, small circular molecules of double stranded DNA of a few thousand base pairs in length. Plasmids are found in bacteria separate from the bacterial chromosome and can often replicate within a single bacterial cell.

Human genes

As a rule, nucleated diploid cells contain two copies of each particular gene, one inherited from each parent. Each gene occupies the same position relative to other genes at one particular place ('locus') on one particular chromosome. The sequence of bases coding for a single polypeptide is not, as used to be thought, continuous but is divided into two or more regions called exons which are separated from one another by non-coding regions called introns (Fig. 1.3). Upstream (5′) to the first

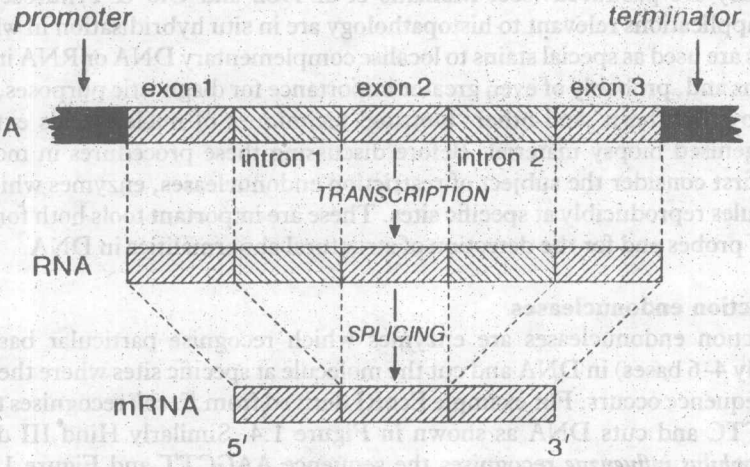

Fig. 1.3 Structure of a typical mammalian gene and its RNA transcript before and after splicing.

exon of the gene, the DNA has a special non-coding region called a promoter which may permit or prevent access of RNA polymerase to that particular stretch of DNA and hence determine whether or not the gene will be transcribed. The 3′ end of the final exon has a termination signal which indicates the point at which RNA polymerase detaches from the chromosome and transcription ceases. In addition to the activity of the promoter region the rate of transcription of some genes is influenced by so-called 'enhancer' sequences which may lie within the gene or some distance upstream or downstream.

The genes account for only a small fraction of nuclear DNA, the remainder being composed of non-coding DNA which is never transcribed and whose function is largely unknown. In contrast to the genes, nearly all of which are present as single copies (two copies in diploid cells), many copies of certain non-coding sequences are widely distributed at fixed points throughout the chromosomes (repetitive sequences).

Post transcriptional mRNA processing

When a gene is active both exons and introns are transcribed into RNA but the parts corresponding to the introns are later removed and the ends of the remaining exons are spliced together to form mRNA before translation begins. In addition, a poly A sequence (AAAAA.....) is usually added to the 3′ terminal of the mRNA molecule — the 'poly A tail' which is useful for identifying and purifying mRNA in recombinant DNA technology.

METHODS IN RECOMBINANT DNA TECHNOLOGY

For the histopathologist the most important aspect of recombinant DNA technology is the production of labelled DNA and RNA probes for the study of nucleic acids in samples of diseased tissue. Although labelled probes for use in histopathology will mostly be obtained from research laboratories and firms specialising in biotechnology, the pathologist should have a knowledge of the nature of such probes and the way they are produced (see, Maniatis et al 1982 and Old & Primrose 1985). The main applications relevant to histopathology are in situ hybridisation in which labelled probes are used as special stains to localise complementary DNA or RNA in histological sections and, probably of even greater importance for diagnostic purposes, the examination by 'Southern' and other 'blotting' techniques of nucleic acids extracted from homogenised biopsy material. Before discussing these procedures in more detail we must first consider the subject of restriction endonucleases, enzymes which cut DNA molecules reproducibly at specific sites. These are important tools both for the production of probes and for the detection of structural abnormalities in DNA.

Restriction endonucleases

Restriction endonucleases are enzymes which recognise particular base sequences (usually 4–6 bases) in DNA and cut the molecule at specific sites where the appropriate base sequence occurs. For example EcoRI derived from *E. coli* recognises the sequence GAATTC and cuts DNA as shown in Figure 1.4. Similarly Hind III derived from *Haemophilus influenzae* recognises the sequence AAGCTT and Figure 1.4 illustrates the cutting of DNA by this enzyme. More than four hundred different restriction

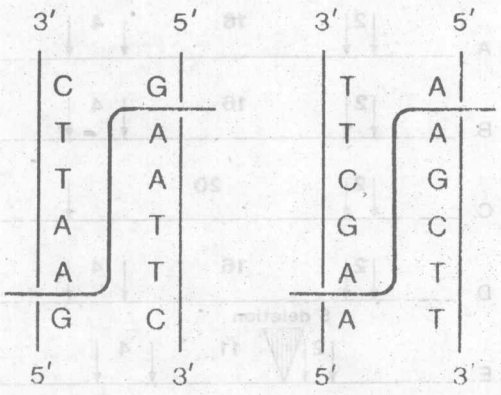

Fig. 1.4 Cutting of double stranded DNA molecule and cleavage of recognition sequences of double stranded DNA by restriction endonucleases EcoRI (left) and Hind III (right). The staggered cleavage creates 'sticky' ends which tend to reunite.

endonucleases are now available. The ability to cut DNA at specific sites has many practical implications three of which are especially worthy of mention.

Formation of recombinant DNA molecules

When the cuts in double stranded DNA are staggered, i.e. not directly opposite each other (Fig. 1.4), the end of each fragment consists of a 'sticky' single stranded tail which tends to adhere to the complementary sticky ends of other DNA fragments produced by the same enzyme. Thus, if fragments of human DNA made with EcoRI are mixed with plasmid DNA predigested with the same enzyme, recombination is likely to occur between some of the human and plasmid DNA to form hybrid molecules. These may still retain the ability of the original plasmid to replicate in bacteria and, in this way, a large number of copies of recombinant human DNA fragments may be obtained for use in preparing probes.

Restriction fragment length polymorphism

Identical chromosomes have identical base sequences and they are cut by restriction endonucleases at identical points to produce identical sets of restriction fragments. Structural differences between the DNA from different individuals can often be demonstrated by differences in restriction fragments. Figure 1.5 is a hypothetical example illustrating part of a chromosome (say the X chromosome) from five different men and showing the restriction sites cut by one of the endonucleases such as Hind III. Notice that the restriction sites in individuals A, B and D are at identical positions and that the lengths of the three fragments produced are the same. In Mr C's X chromosome one of the restriction sites is absent, perhaps due to a change in the base sequence at the site by a point mutation (one base replaced by another) or by a small deletion removing part or all of the site. In Mr E all the sites are present but the 16 kilo-base (kb) pair fragment has been reduced to 11 by a 5 kb deletion which has not included a Hind III restriction site. The size of restriction fragments can readily be measured by electrophoresis in agarose gel (the smaller the fragments

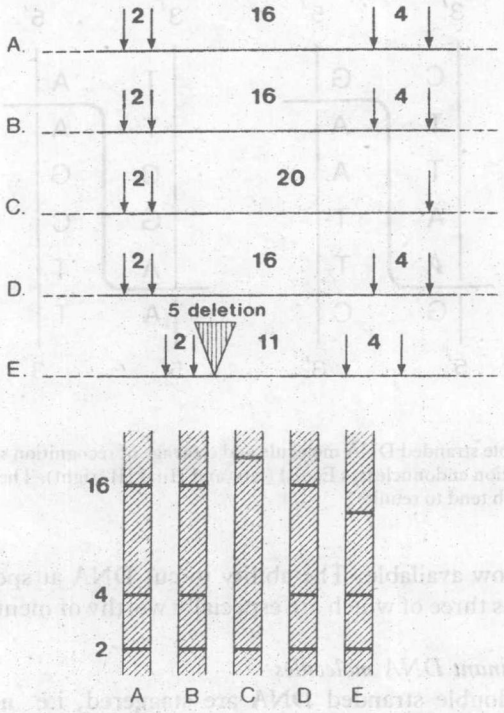

Fig. 1.5 Hypothetical restriction map of part of the X chromosome from five males. Above: horizontal lines represent DNA molecules which are cut by a restriction enzyme at points marked with arrows. Numbers represent lengths of restriction fragments in kilobases. Individuals A, B and D have identical restriction maps with this enzyme. The others illustrate restriction fragment length polymorphisms, C due to a point mutation abolishing one of the restriction sites and E due to a deletion in the largest fragment between two adjacent restriction sites. Below: agarose gel, electrophoresis of restriction fragments illustrated above; the polymorphism between individuals is readily apparent.

the further they migrate) and it is thus possible in principle to detect three polymorphisms in the part of the X chromosome illustrated from these five men.

Similar methods can be used to compare DNA from normal and neoplastic tissues and studies of this kind have led to the discovery of qualitative abnormalities in or near genes which may be of fundamental importance in normal growth and development and in carcinogenesis.

Analysis of recombinant DNA molecules

The electrophoretic examination of restriction fragments is an essential step in the characterisation of DNA probes prepared by recombination between human and plasmid DNA.

Production of DNA probes

The use of a plasmid to clone a potential probe consisting of a restriction fragment of human DNA is illustrated in Figure 1.6. In this example the same restriction enzyme is used to prepare the fragment of human DNA and to cut open the plasmid. Because they have complementary sticky ends, recombination sometimes takes place

to form a closed circle composed of human and plasmid DNA which has the property of acting as a carrier or vector by infecting and replicating in bacterial cells. Following culture, lysis of the infected bacteria releases large numbers of recombinant plasmids. After labelling, the whole recombinant plasmid may be used as a probe or the human sequences may be excised with the appropriate restriction enzyme and purified by electrophoresis.

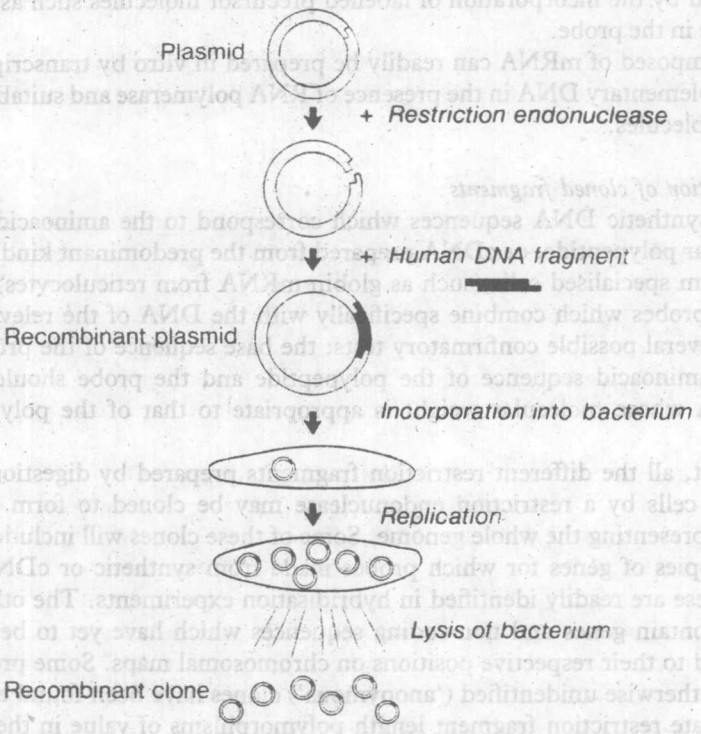

Fig. 1.6 Cloning of a restriction fragment of human DNA in a plasmid.

Instead of restriction fragments as in the above example, probes may be constructed with cDNA copies of mRNA transcripts or from small pieces of DNA synthesised in accordance with the known aminoacid sequence of some polypeptide.

The plasmids called pBR322 and pAT153 are often used in recombinant DNA technology. They have been specially constructed with suitable restriction sites for the insertion of foreign DNA into marker genes (e.g. for antibiotic resistance) which indicate if recombination has occurred and permit the selection of probe-producing bacterial colonies in culture. Sometimes the bacterial virus, phage lambda, which can accommodate larger inserts of human DNA is used as a vector instead of a plasmid.

Labelling techniques

The radioisotope ^{32}P is the usual label for probes used in blotting techniques while ^{3}H (tritium), which has a shorter track length in photographic emulsions and therefore allows more precise localisation at the histological level, is preferred for in situ hybridi-

sation studies in tissue sections. Probes required for the detection of DNA or RNA sequences that are only present in small amounts (e.g. one or two copies per cell) must be highly radioactive and unfortunately pose serious health and disposal problems which preclude their widespread use in routine tests. Non-radioactive labels are being developed and some of them (e.g. biotin plus alkaline phosphatase-labelled streptavidin) are now approaching the radioactive labels in sensitivity. Labelling is generally accomplished by the incorporation of labelled precursor molecules such as adenosine triphosphate in the probe.

Probes composed of mRNA can readily be prepared in vitro by transcription from cloned complementary DNA in the presence of RNA polymerase and suitably labelled precursor molecules.

Characterisation of cloned fragments

Cloning of synthetic DNA sequences which correspond to the aminoacid sequence of a particular polypeptide, or cDNA prepared from the predominant kind of mRNA obtained from specialised cells (such as globin mRNA from reticulocytes) are likely to produce probes which combine specifically with the DNA of the relevant genes. There are several possible confirmatory tests; the base sequence of the probe should reflect the aminoacid sequence of the polypeptide and the probe should combine with mRNA whose molecular weight is appropriate to that of the polypeptide in question.

In contrast, all the different restriction fragments prepared by digestion of DNA from whole cells by a restriction endonuclease may be cloned to form a 'library' of probes representing the whole genome. Some of these clones will include complete or partial copies of genes for which probes made from synthetic or cDNA already exist and these are readily identified in hybridisation experiments. The other cloned fragments contain genes and non-coding sequences which have yet to be identified and allocated to their respective positions on chromosomal maps. Some probes made from some otherwise unidentified ('anonymous') clones have been found empirically to demonstrate restriction fragment length polymorphisms of value in the diagnosis and prevention of genetic diseases.

In situ hybridisation techniques

The in situ demonstration of specific DNA or mRNA sequences in tissue sections or cytological preparations can be achieved with probes consisting either of DNA or mRNA. 'Antisense' RNA is the preferred probe for localising mRNA since the use of a labelled 'sense' strand as a negative control on another slide provides a valuable test of specificity.

The techniques for demonstrating nucleic acids in tissue sections by in situ hybridisation follow the same general pattern as those for immunohistological staining of soluble antigens with labelled antibodies. Both depend on prior fixation of the tissue to preserve adequate morphological detail and to immobilise the target substance in an insoluble form which still retains its specific reactivity. In each procedure the section is treated with a solution of the appropriate reagent — labelled antibody or probe — then washed, and the labelled molecules which remain bound to the tissue are rendered visible for microscopic examination.

Formalin fixation is satisfactory for in situ hybridisation of specific mRNA in tissue