necessary self examination that provokes a major change in the direction of your professional life. If so, so be it. It is good that such changes should be made while time is still on our side. Otherwise we are in danger of joining what Thoreau thought to be the majority of men, who "lead lives of quiet desperation." So if you haven't already done so start planning your sabbatical now—and don't forget to tell your partners and your spouse.

Useful contacts for sabbatical employment

Bureau for Overseas Medical Service (BOMS)
Africa Centre, 38 King Street, London WC2 8JJ
Telephone: 01 836 5833
Administrator: Jane Lethbridge

Christians Abroad
11 Carteret Street, London SW1H 9DL
Information secretary: Deborah Padfield

Action Health 2000
35 Bird Farm Road, Fulbourne, Cambridge CB1 5DP
Telephone: 0223 245252 ext 7466 (2-5 pm)
Director: Dr M Kapila

Overseas Development Administration
Crown Agents, 4 Millbank, London SW1P 3JD
Inquiries to the recruitment executive

Voluntary Service Overseas (VSO)
9 Belgrave Square, London SW1X 8PW

International Voluntary Service (IVS)
53 Regent Road, Leicester LE1 6YL

Oxfam
274 Banbury Road, Oxford OX2 7DZ
Inquiries to the disaster emergency officer

Save the Children Fund
Mary Datchelor House, 17 Grove Lane, Camberwell, London SE5 8RD

British Red Cross Society
9 Grosvenor Crescent, London SW1 7ET

Basic Molecular and Cell Biology

Methods in molecular medicine

R K CRAIG

During the past decade an array of powerful new diagnostic techniques has been developed based on nucleic acid hybridisation and gene probes. These allow the direct analysis of genes in deoxyribonucleic acid (DNA) extracted from the nuclei of human cells or, alternatively, of gene transcripts in the form of messenger ribonucleic acid (mRNA), the template for protein synthesis found in the cytoplasm. These techniques contrast with the use of antibodies, which permit the analysis of the gene product or protein, either in the cell or in cell secretions—for example, plasma (fig 1).

The concept of hybridisation

DNA is made up of four building blocks or bases: adenine (A), guanine (G), cystosine (C), and thymine (T). Within a strand of DNA the bases are linked by a sugar-phosphate backbone. Within the cell the DNA is in a highly ordered double stranded helical structure. The helical structure is maintained through specific hydrogen bonding interactions between complementary bases, so that A in one strand always pairs, or hybridises, with T in the other and C hybridises with G. Thus A and T and C and G are termed complementary bases and must always be present in equivalent amounts in double stranded DNA.

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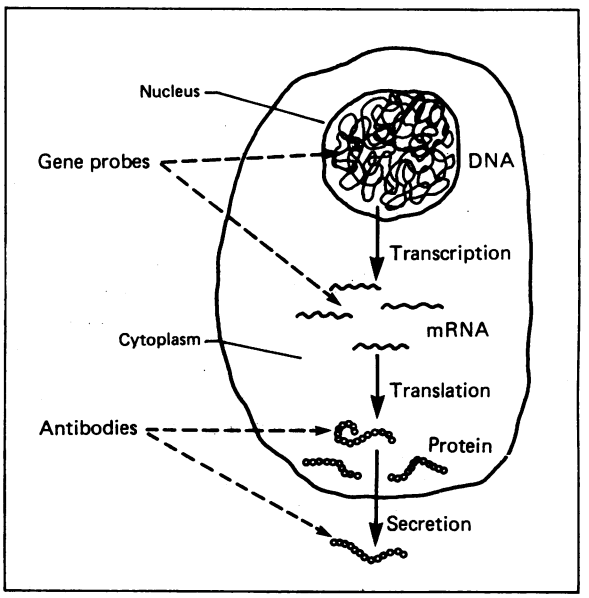

FIG 1—Site of action of gene probes.

A region of DNA which encodes a protein is termed a gene. The genetic information is encoded by the sequence of bases via a non-overlapping code in which three bases (a triplet) determine a particular amino acid (see reference 1 for well illustrated reading). For a gene to be expressed an enzyme, RNA polymerase II, copies or transcribes one strand of the DNA into mRNA, which is then decoded or translated by the protein synthesis machinery in the

cytoplasm. The mRNA comprises a single stranded polynucleotide chain with a sugar-phosphate backbone in which the order of bases is the complement of the transcribed DNA strand of the gene. In RNA thymine (T) is replaced by a closely related base uracil (U), which also will base pair with, or hybridise to, adenine (A).

When DNA is isolated from cells or tissues it is usually recovered in a double stranded or native form. If a solution of DNA is heated or placed in an alkaline environment, however, the hydrogen bonds between the bases are broken and the strands separate, giving rise to denatured or single stranded DNA. If a heat treated denatured DNA solution is permitted to cool very slowly then the complementary strands will again base pair or hybridise, resulting in the formation of native double helical DNA, indistinguishable from the original native preparation (fig 2). It is the ability of long stretches of one strand of denatured DNA, containing infinitely variable combinations of the four bases, to hybridise in a precise manner to its complementary strand that forms the basis of nucleic acid hybridisation technology. Thus, as we shall see, radiolabelled single stranded RNA or DNA sequences, complementary to the gene sequence of interest, can with great precision be used to identify the presence of individual genes in DNA preparations and specific mRNA in RNA preparations, or even within individual cells.

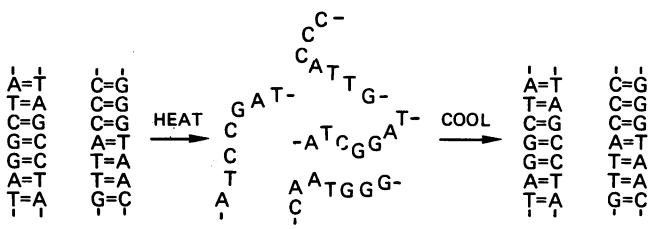

FIG 2—Effect of heating in separating double stranded DNA and of slow cooling in hybridising the strands to form double stranded DNA indistinguishable from the original.

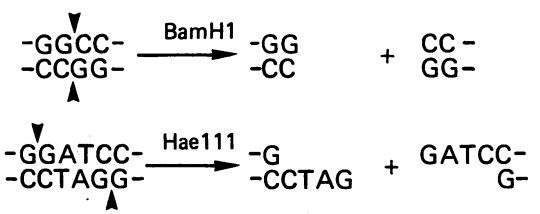

FIG 3—Sequences cleaved by two restriction enzymes, Hae 111 and Bam H1.

Looking at genes

The potential coding capacity of DNA in a single human cell is enormous. In each diploid cell double stranded DNA is distributed between 23 pairs of chromosomes, comprising about $3{\cdot}5\times10^9$ base pairs, or two metres of DNA if stretched end to end. Potentially this might encode 2 million genes. In reality half of the DNA is composed of short simple sequences repeated many times—for example, (ACAAACT)n, termed satellite DNA. These sequences have no obvious function. Of the remainder, some comprise more complex sequences, which occur many times but at different points in the genome. The rest, the so called unique sequences, or single copy genes, represent long tracts of sequence many kilobases (1000 bases) long, each of which occurs perhaps only once in each haploid cell. These encode somewhere between 50 000 and 200 000 different protein products.

The analysis of a single gene within the vast amount of genetic material in a cell depends on the following principles. Firstly, the long and unmanageable human DNA isolated from cells and tissues must be cut into smaller but reproducible pieces. Secondly, hybridisation probes specific for individual genes must be generated.

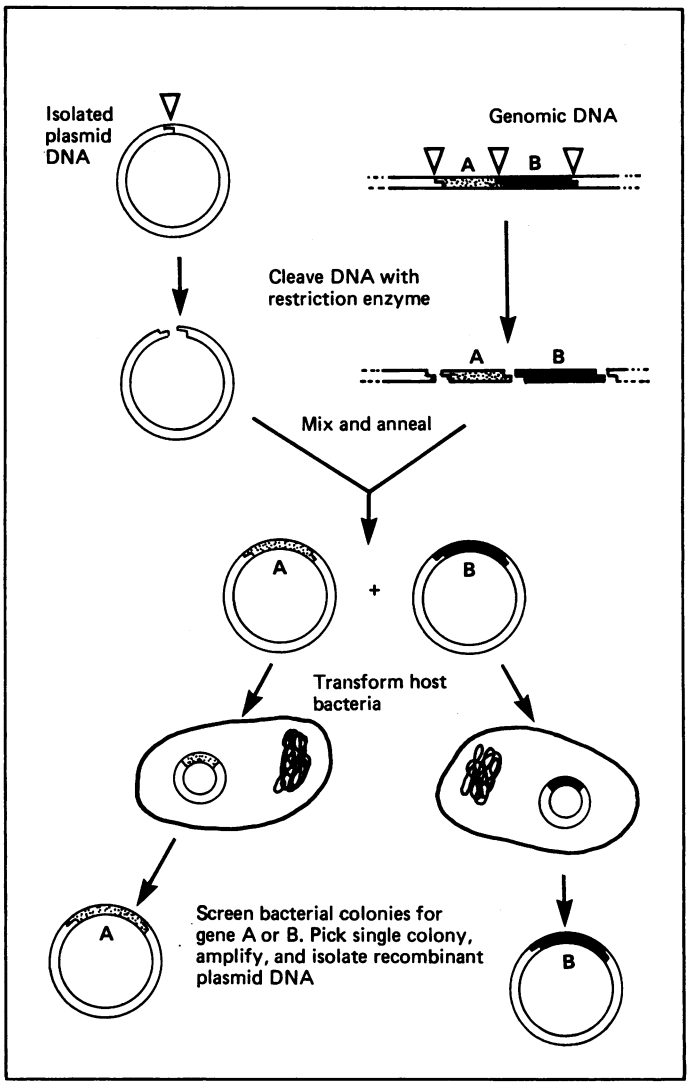

FIG 4—Generation of hybridisation probes by cleavage of high molecular weight DNA with restriction enzymes, followed by insertion of the individual fragments into bacterial plasmids.

Thirdly, techniques are required which permit the direct visual comparison of, for instance, single copy genes from different individuals or genes from normal or cancerous tissue from an individual.

The identification of a class of endonucleases (restriction endonucleases) which cleave DNA at specific sites determined by a short sequence of bases provided molecular scissors. With these a solution of native DNA, representative of DNA isolated from many millions of cells, can be cut so that the DNA from each cell is cleaved into an identical set of fragments. Different restriction enzymes cut double stranded DNA at different recognition sequences and therefore cleave a given DNA preparation into different fragments of different lengths characteristic of the enzyme. Some enzymes recognise a sequence of four bases—for example, Hae III—others five or six bases—for example, Bam HI. Enzymes recognising four bases will cleave the DNA more often than those recognising five or six bases. DNA fragments can then be separated electrophoretically on the basis of size and the relative position of a specific gene determined by hybridisation (see below).

The molecular cloning and characterisation of human gene sequences is beyond the scope of this article (for further reading see reference 2), but large numbers of bacterial plasmids, each containing part of the human genome, may be constructed (see fig 4). Individual plasmids containing any desired gene may be selected from a "gene library" and the cloned or inserted gene sequence of interest characterised by DNA sequence analysis. Each characterised plasmid containing a selected gene sequence may then

be grown (amplified) in bacterial host cells and large amounts of plasmid DNA containing the inserted gene isolated. This recombinant plasmid DNA may then be used as a source of a specific gene probe in subsequent hybridisation analyses. Alternatively, once the nucleotide sequence of a gene is known, a desired sequence of bases may be synthesised chemically—an oligodeoxynucleotide—and used as a hybridisation probe.

The analysis of individual gene structure (gene mapping) in health and disease uses purified DNA isolated from tissues or, more often, peripheral blood lymphocytes. The isolated DNA is treated with a specific restriction enzyme, and the restriction fragments generated are separated on the basis of size by electrophoresis on an agarose gel. The separated DNA fragments are denatured in the gel by alkali treatment and then transferred on to a nitrocellulose membrane using a process called Southern blotting, named after the inventor, Professor E M Southern (fig 5A).

The size of a restriction fragment of DNA, which contains a specific single copy gene, may then be determined by hybridisation analysis using a radiolabelled gene probe complementary to the now denatured gene sequence under investigation. Radiolabelling permits the position of the hybridised gene probe to be pinpointed by autoradiography—on x ray film—and consequently the size of the identified gene fragment can be determined relative to markers separated in parallel by electrophoresis. Thus the Southern blot in figure 5B shows two different sizes of restriction fragment containing the single copy human calcitonin gene after digestion of human DNA with two different restriction enzymes. Cleavage of the genomic DNA with different restriction endonucleases permits us to build up a picture (or map) of the distribution of restriction enzyme cleavage sites within the vicinity of specific genes in the human genome. Southern blots may also be probed with sequences which occur many times and at different parts of the genome. These repeated or "minisatellite" sequences permit the visualisation of different parts of the genome on a single gel. Minisatellite probes provide a powerful means of detecting sequence differences in the genome of individuals, the differences generally reflecting the presence or otherwise of recognition sites for specific restriction enzymes (see fig 5C). Minisatellite probes have made a significant impact on forensic medicine (see reference 3).

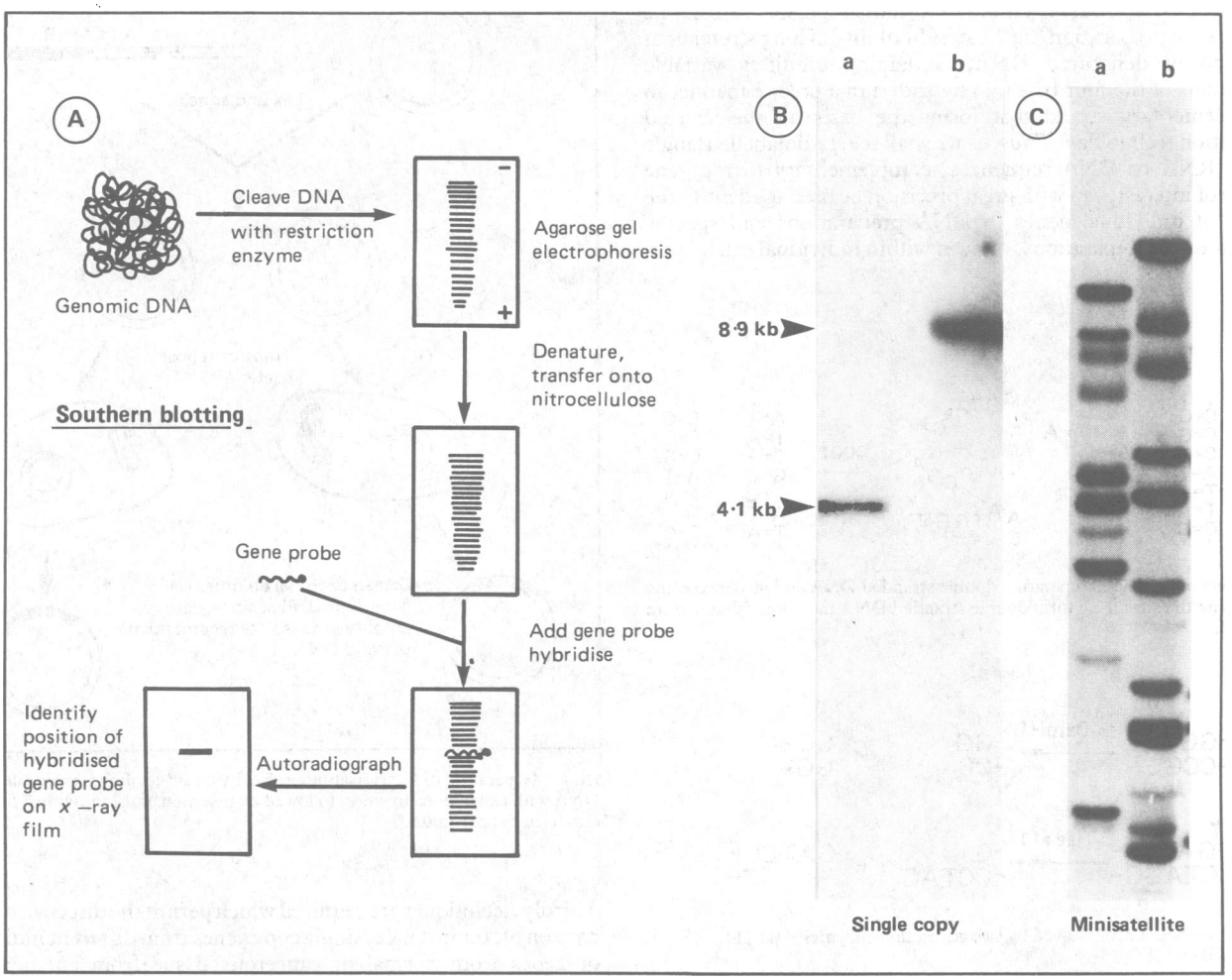

FIG 5—Looking at genes. (A) Southern blotting. (B) Identification of restriction fragments of human genomic DNA containing the calcitonin gene after digesting genomic DNA isolated from a single individual with the restriction enzyme (*a*) Pst 1 or (*b*) Hind 111. Fragments were Southern blotted and those containing the calcitonin gene identified by hybridisation using a calcitonin specific gene probe. (C) Genetic fingerprints of two unrelated individuals (*a* and *b*) after digestion of their genomic DNA with the restriction enzyme Hinf 1, Southern blotting, and hybridisation with a "minisatellite" gene probe.

Looking at gene expression

The technique of hybridisation analysis using radiolabelled cloned gene probes may also be used to identify specific RNA transcripts within cells and tissues. Total cellular RNA may be isolated from cells in culture or fresh or frozen tissue and the RNA species separated on the basis of size by gel electrophoresis and then transferred to a nitrocellulose membrane by blotting. RNA, or "Northern," blotting follows the same principles as DNA blotting, except that there is no need for denaturing (RNA is single stranded) or cleaving with restriction enzymes. The presence and size of a

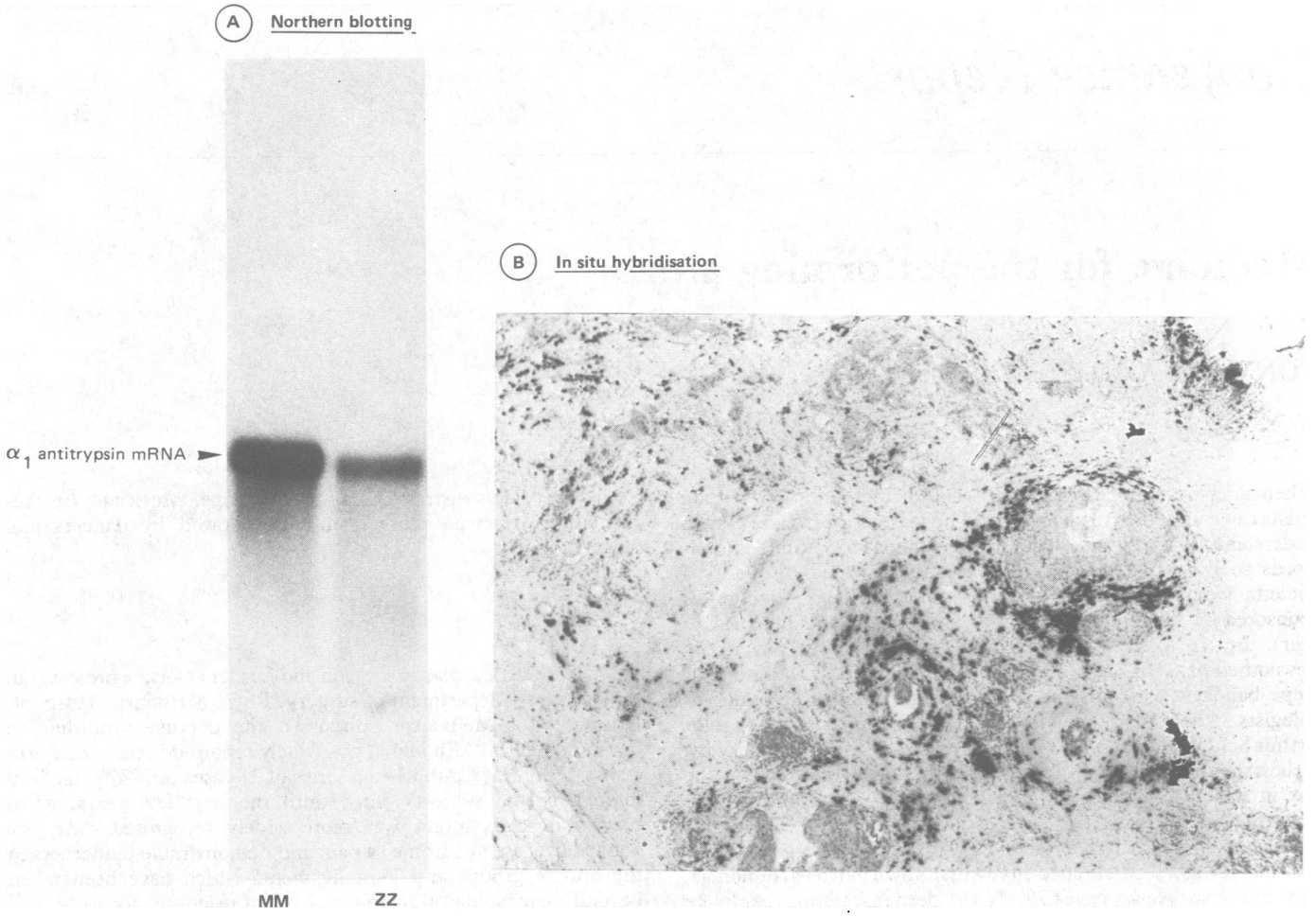

FIG 6—Looking at gene expression. (A) Comparison of α_1 antitrypsin mRNA levels in total liver RNA from individuals with MM and ZZ phenotypes by Northern blotting. Each tract represents 5 μg of RNA, blotted, then hybridised using an α_1 antitrypsin gene probe. (B) Identification by in situ hybridisation of K chain mRNA producing B cells in a formalin fixed, paraffin embedded section of an infiltrating duct breast carcinoma.

specific mRNA species is then determined using a radiolabelled hybridisation probe followed by autoradiography. Northern or RNA blotting can be used to determine the presence or absence of mRNA species and the relative amount of these mRNAs in normal and diseased states—for example, the relative amounts of α_1 antitrypsin mRNA present in RNA from the liver of normal (MM) or α_1 antitrypsin deficient (ZZ) individuals (fig 6A).

Northern blotting will not, however, provide information on which cells within a tissue are expressing a gene of interest. This requires a technique analogous to immunocytochemistry, known as in situ hybridisation (see reference 4). This entails the hybridisation of radiolabelled gene probes to the complementary mRNA species in frozen or paraffin embedded fixed tissue sections. After hybridisation the sections are dipped in photographic emulsion and stained. Cells which express the gene under investigation are then identified by the presence of silver grains localised over the cell. The example shown (fig 6B) identifies infiltrating B cells synthesising immunoglobulin light chain (K) mRNA in a formalin fixed, paraffin embedded section of a breast tumour.

Conclusion

The power and sensitivity of the technology now available to those who want to apply molecular techniques in clinical research are remarkable. Much of the technology can now be classed as routine. Characterised gene probes are readily available, as are kits to radiolabel them. The transfer of the technology from the research to the routine laboratory awaits only the development of detection methods that do not depend on radioisotopes with short half lives and are not so hazardous to handle. Once this limitation has been overcome gene probe kits will become commonplace, applicable in all branches of diagnostic medicine.

References

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