

MOLECULAR BIOLOGY IN CLINICAL HAEMATOLOGY

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Molecular biological techniques have made dramatic changes in the diagnosis and management of many haematological disorders. Great strides in understanding the pathogenesis of thalassaemias, haemoglobinopathies, inherited haemolytic anaemias, leukaemias, lymphomas and coagulation disorders, have been made by the application of techniques highlighted in the article by Dr. R. Trent. This article will attempt to provide a brief overview of the "cutting edge" of haematology.

Thalassaemias and Haemoglobinopathies

Haemoglobin has long been a focus of research in molecular biology for the simple reason that red cells, which are so easily available, contain large quantities of that single protein. Immature red cells actively manufacture haemoglobin and therefore contain large quantities of messenger RNA specific for globin chains, that can be easily isolated, studied and more importantly be used to manufacture complementary DNA (cDNA) which can then be cloned for use as probes in diagnostic tests. The normal haemoglobin molecule consists of two alpha and two beta chains – genes for these are located on chromosome 16 and 11 respectively. The alpha gene occurs in two copies and the beta gene occurs only as a single copy. This simple numerical difference alone explains some of the major differences in the clinical manifestations of the two types of thalassaemias. Should one alpha gene be lost or mutated the other can take over the function. A single mutated alpha gene causes no clinical symptoms (2 trait) and even in individuals homozygous for the trait (1 trait) there are no serious symptoms as they have two more functioning genes. In patients with three genes missing or malfunctioning (Hb H disease) there is still a good life expectancy. However, with the beta chain genes there is no "backup" gene and therefore any abnormality to this single gene will have more serious consequences.

Abnormalities are detected by the techniques discussed by Dr. J. Trent i.e. restriction enzyme analysis of DNA and looking for differences between patterns of normal and test samples as seen in an autoradiograph (Fig. 1). Even a point mutation viz. as seen in sickle cell anaemia can be detected if an appropriate restriction enzyme is used. This restriction enzyme is chosen on the basis of the mutated base i.e. its "substrate site".

Studies like the above have led to a classification of thalassaemias according to the site of molecular pathology. The following list summarizes abnormalities affecting the globin genes, their translation and transcription, all of which lead to various clinical manifestations:

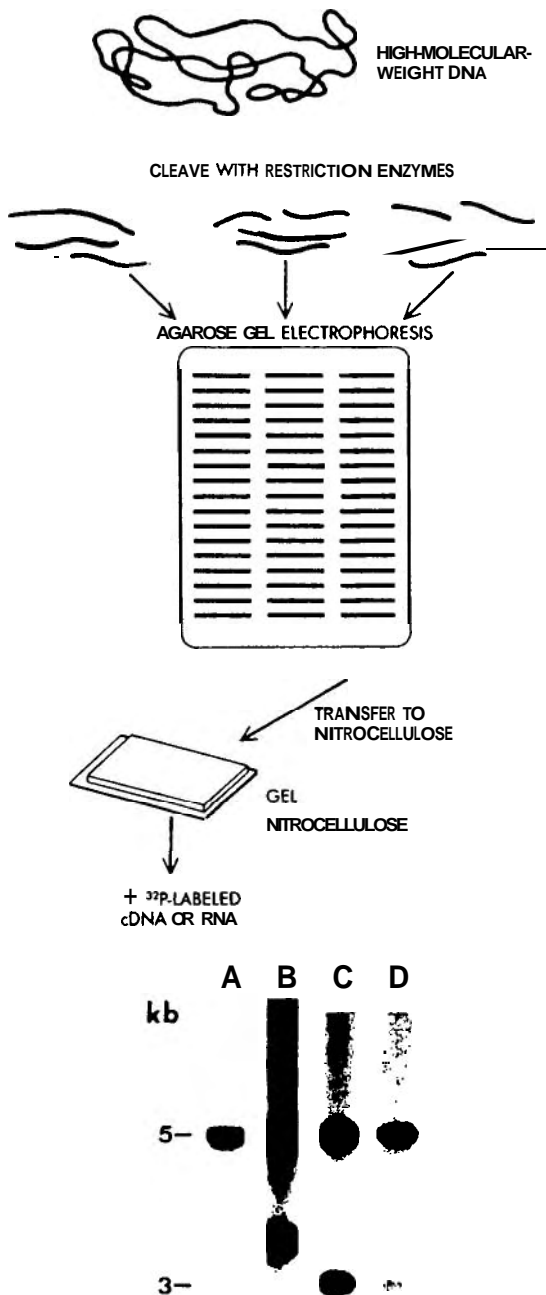
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|----------------------------------|---|---|
| a) mutation of splice site | } | Changes affecting the reading of the mRNA message |
| b) loss of normal terminator | | |
| c) frameshift mutation | | |
| d) posttranslational instability | | |
| e) gene deletions | } | Abnormalities affecting the gene itself |
| f) gene mutations | | |
| g) promotor site mutations | | |

Some of these abnormalities are more commonly seen with alpha-thalassaemias and others are associated with beta-thalassaemias. Also certain abnormalities are associated with different ethnic groups. For more details, the reader is referred to Nienhuis *et al.*

Inherited Haemolytic Anaemias

The red cell membrane skeleton is a highly organized network of protein including spectrin, actin, protein 4.1 and ankyrin. The red cells lose their characteristic shape and become susceptible to fragmentation when the integrity of the membrane is disrupted by structural abnormalities or deficiencies of its protein components. Recently the molecular basis for a subset of hereditary elliptocytosis was described. The study located the gene of protein 4.1

Fig. 1. The Southern Blot Procedure for the demonstration of the presence or absence of DNA rearrangements



1. A blood sample is taken from the patient and the nuclei of the white cells are obtained.
2. The DNA is isolated from the protein components of the nuclei and quantitated.
3. The isolated DNA is digested by Restriction Enzymes which cleave DNA in specific sites and generate unique DNA fragments.
4. The DNA fragments of a digested sample are subjected to electrophoresis in an agarose gel where they are separated by fragment size.
5. This size-fractionated DNA is transferred by "Southern Blotting" onto a nitrocellulose membrane by diffusion of a high salt solution.
6. A cloned DNA probe, homologous in DNA sequence to the gene of interest is made radioactive by labelling with ^{32}P - this can be a beta-globin gene, Ig gene, oncogene or Factor IX gene.
7. The radioactive probe is hybridized to the nitrocellulose bound homologous DNA.
8. An autoradiograph is prepared. X-ray film is placed adjacent to the Southern blot. The radioactivity exposes the film at the site of probe attachment of the fragmented DNA. The specific patterns demonstrated on the autoradiograph will indicate the presence or absence of gene rearrangements. viz. if LANE A & D is normal then B, and C are abnormal.

to the short arm (p) of chromosome 1 and showed that the mutant gene had a DNA rearrangement upstream from the initiation codon. This led to an abnormal splice mechanism affecting the mRNA. Abnormality of the spectrin molecule has been found in hereditary spherocytosis, but the ultimate genetic lesion in autosomal dominant HS is still unknown.

Oncogenes and Leukaemias

What are oncogenes?

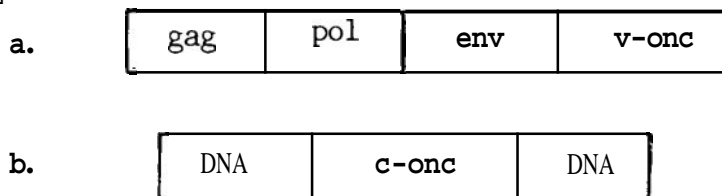
The recent discovery of cellular oncogenes has provided researchers with important clues regarding the origin of neoplasia. Oncogenes derived from RNA viruses were first found to cause cancer in animals such as chickens and mice. This led investigators to suspect a viral aetiology in neoplasia. Upon close examination of neoplastic and normal cell samples, it was discovered that the oncogenes of viruses (v-onc) are almost exact copies of genes in normal

cells (c-onc). These normal cellular genes (called proto-oncogenes) code for protein products which are essential for the orderly proliferation and development of cells. Changes in these genes such as a DNA rearrangement (for example, a chromosomal translocation) or a mutation, alter them from a normal proto-oncogene to a cancer-causing gene called an oncogene (Fig. 2).

This alteration results in the production of an abnormal protein product which leads to loss of normal regulation of cell growth: cancer. There are four main types of oncogenes organized by the function of their protein products: (i) Enzymes which bind to cellular membranes and have receptors for growth factors. (ii) Growth factors. (iii) Enzymes found in the cytoplasm. (iv) Proteins which are bound to the nucleus and are involved in cell division.

Approximately twenty oncogenes have been discovered and each has been designated by a

Fig. 2



a. A genetic map of a typical retrovirus. The gag gene specifies a protein which forms the vital core, pol codes for reverse transcriptase, required for replication of the viral genome, env specifies the glycoproteins of the viral envelope. The fourth gene v-onc is the oncogene responsible for neoplastic transformation in the host.

b. The c-onc gene is a normal component of the human genome putatively responsible for orderly proliferation and differentiation of the cells.

three-letter name. For example, "abl" is the oncogene first noted in Abelson Murine Leukemia Virus. One can indicate the viral oncogene by writing "v-abl" and its cellular counterpart by "c-abl". Some important oncogenes known to be involved in human cancer are: abl (Chronic Myelogenous Leukemia), ras (Erythroleukemia), sis (Sarcoma), erb (Erythroblastosis), myc (Carcinoma, Sarcoma, Myelocytoma) and myb (Myeloblastosis, Erythroblastosis).

Chronic Myelogenous Leukaemia and abl Oncogene

In 1960 Nowell and Hungerford reported the discovery of what at first seemed to be a "minute" deleted chromosome 22 (the Philadelphia chromosome) which has been reported to be present in 90 - 95% of Chronic Myelogenous Leukemia (CML) patients. Later, this abnormally sized chromosome was actually found by Rowley to be the result of a reciprocal translocation between 9 and 22, resulting in a net gain of genetic material on 9 and a net loss on 22.

In 1970, H.T. Abelson reported a Retrovirus-inducing disease in mice which is now known as Abelson Murine Leukemia Virus and Abl Mu L V. De Klein and co-workers used a v-abl probe derived from this virus and demonstrated that the homologous cellular oncogene is located on chromosome 9 but not on 9q+, and on 22q- but not on 22. This indicated that the

c-abl sequence must be translocated from 9 to 22 in the Philadelphia translocation. This was further supported by experiments which clearly demonstrated that c-abl was translocated to chromosome 22. It was postulated then that the translocation transformed the c-abl proto-oncogene to an oncogene.

The bcr gene is discovered

Researchers began to look very carefully at chromosome 22 particularly, at the site to which c-abl was being translocated. Croffon and co-workers studied 17 CML patients and mapped their Philadelphia breakpoints (the rearranged chromosome 22). All of the breakpoints were localized to a 5.8 kilobase sequence. This region was designated the "breakpoint cluster region" or "bcr" of chromosome 22, the site at which chromosome 22 almost always breaks and the site to which c-abl is translocated (Fig. 3). Amazingly, bcr was found to be a gene, able to code for a protein, which has not yet been identified. The translocation of bcr can be detected by molecular methods (Fig. 4).

The hybrid abl-bcr gene

The significant event occurring in the Philadelphia translocation is a rearrangement leading to the juxtaposition of c-abl and the 5' part of bcr, because it was found to code for an abnormal m-RNA which is translated into an abnormal protein. Thus, a hybrid protein is

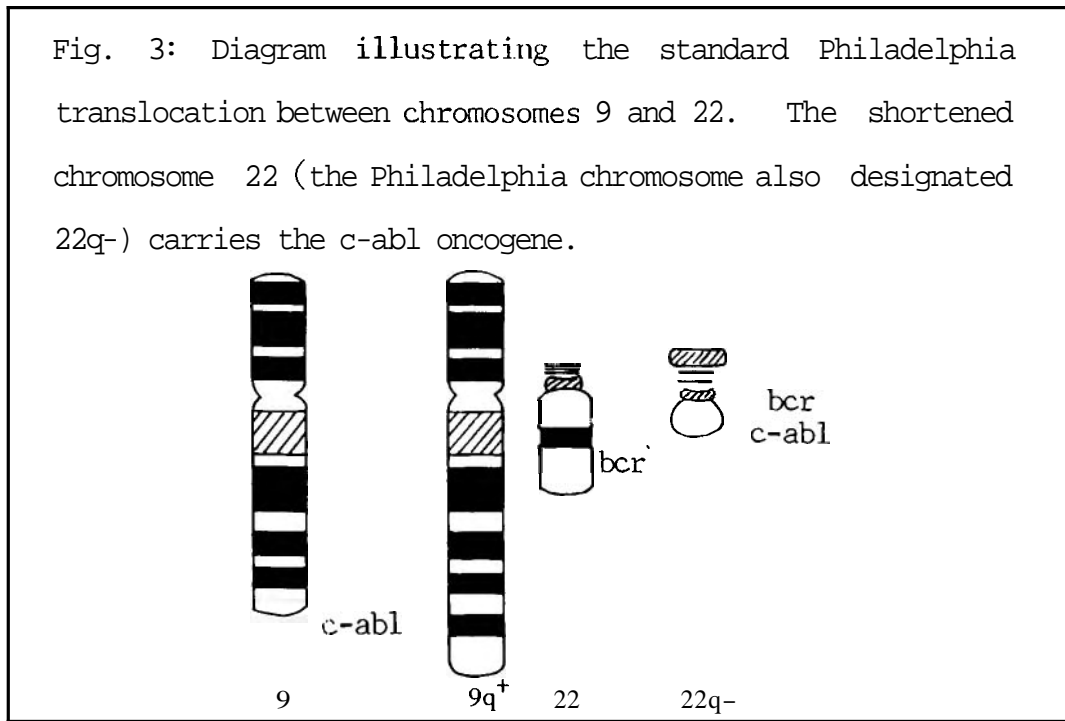
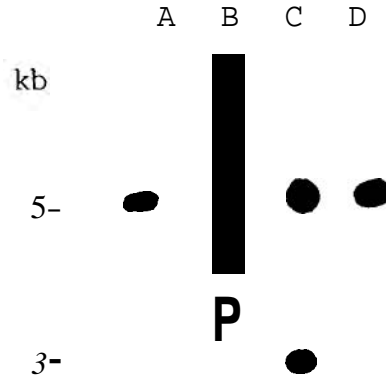


Fig. 4: Diagrammatic presentation of autoradiograph of a Southern Blot analysis of the bcr-abl juxtaposition using a bcr probe.

- A. Normal human placental DNA;
 B. CML patient DNA
 C. CML patient DNA
 D. CML patient DNA after initiation of therapy*



* Note presence of only one band, a normal 5 kb DNA fragment. The different band patterns indicate changes in the gene location i.e., it has been translocated to a new site. Thus this is a molecular means of detecting the Philadelphia chromosome.

generated which is composed of a sequence coding for part of the bcr gene product at one end and part of the normal c-abl product at the opposite end! This protein has been shown to demonstrate high levels of protein tyrosine kinase activity. Since translocation involves the breakage of a chromosome and physical attachment to a new site on a different chromosome, then it is apparent how the transformation of a proto-oncogene to oncogene might occur.

Another oncogene or proto-oncogene which may be of interest to the Haematologist is c-fms for the product of this gene is closely related and probably identical to the receptor for the macrophage colony stimulating factor (CSF-I).

This gene maps to chromosome 5 (5q 33.3) close to that of the CSF-1 gene at 5q 33.6. Deletions of this region of chromosome 5 are

associated with 5q- refractory anaemia syndrome, myelodysplastic syndromes and secondary acute leukaemias.

Restriction Fragment Length Polymorphisms (RFLP'S)

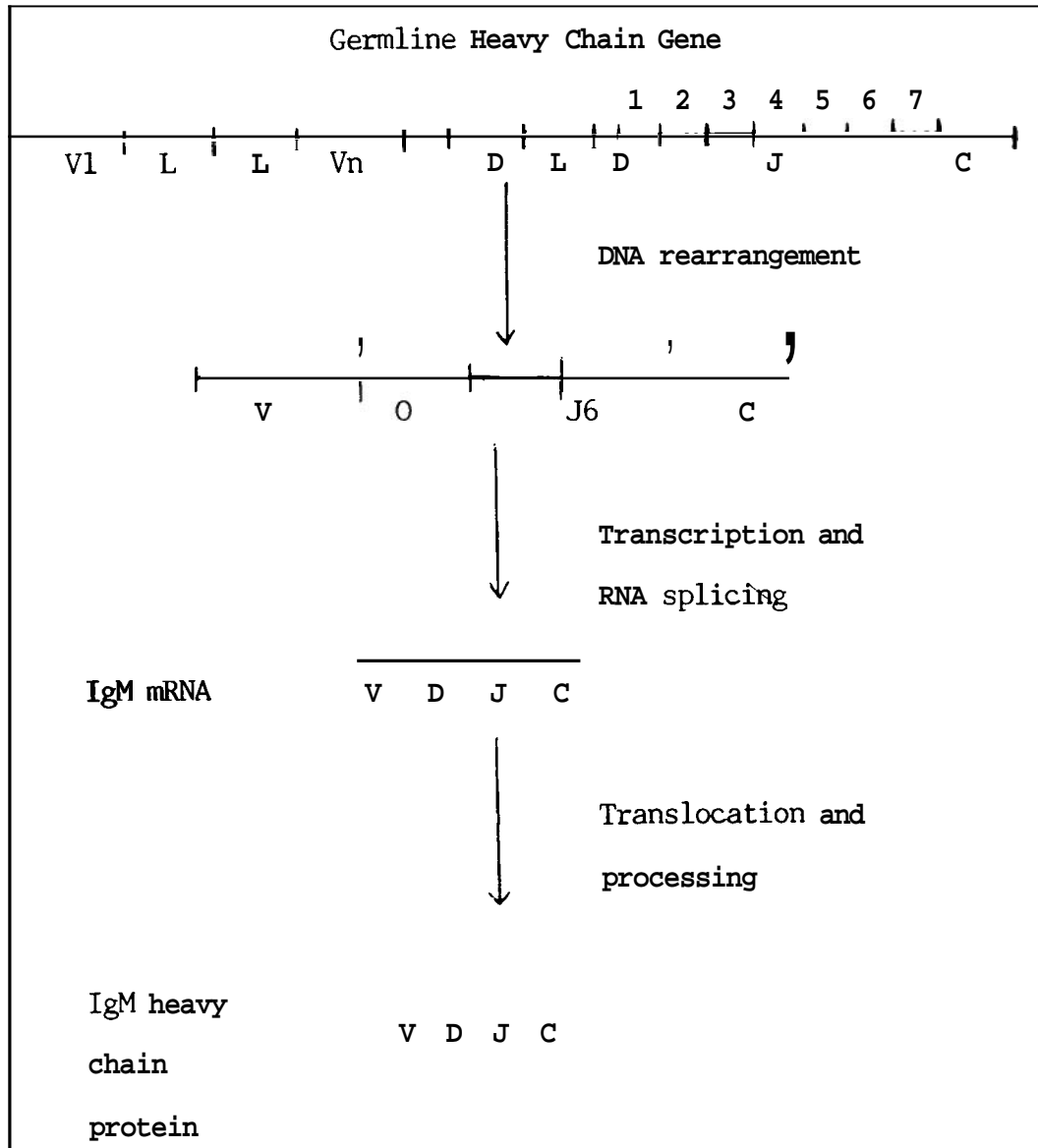
Polymorphisms are defined as genetic differences among individuals. The two possible alleles for restriction endonuclease cleavage site are either (+) i.e. the enzyme cuts at that site or (-) the enzyme does not cut. If the least frequent allele is present in more than 1.1% of individuals, then that particular site is considered to be polymorphic in a given population. The presence or absence of this recognition site determines the sizes of the fragments (RFLP's) which are to be recognised by a specific "probe" i.e. a DNA fragment

which hybridizes to the DNA in the vicinity of the recognition site. **RFLPs** are inherited along Mendelian lines and therefore it is possible to link an RFLP with a particular genetic defect and then follow the defect through a family by mapping for the linked RFLP. An advantage of RFLP approach is the ability to identify abnormal genes even though the molecular basis for that disorder has not been established. The disadvantages are that (i) family and ethnic studies are necessary and (ii) an affected member cannot be excluded from the study. RFLP studies are being used routinely in the studies of patients and families with **thalassaemias** and inherited coagulation disorders.

Gene Rearrangements and Lymphomas

In the last few years much has become known about molecular events in B cells that occur to produce **immunoglobulin** and in T cells that produce the corollary molecule, the T cell receptor. Variable (v), constant (c), diversity (d) and joining (j) region genes code for heavy and light chains of immunoglobulin. These are on chromosome 8, 22, and 9 respectively. Similar gene regions also code for the beta, gamma and alpha chains that make up the T cell receptor. The gene for the beta chain is on the chromosome 7.

These genes are strung out in a particular region of the respective chromosome of all



cells. During B and T lymphocyte development and differentiation a series of recombination events take place, that is shuffling and rearrangement occur, linking directly a v-region with a c-region. This rearrangement is brought about by the deletion of DNA, not the splicing of RNA. The deletion of DNA is a very special and unique characteristic of the rearranged immunoglobulin and T-cell receptor genes of B and T cells respectively and this is the characteristic that makes it possible to detect clonal B and T cells. The DNA rearrangements are detected by using an immunoglobulin gene probe for B-cell and T-cell receptor gene probe for T cells. The rearrangements of the genes alter the location of restriction endonuclease sites and therefore the size of the fragment bearing the genes. These unique rearrangements in a monoclonal population of B cells or T cells can be identified as a unique band on an autoradiograph, compared to a diffuse band produced by a polyclonal population.

The use of immunoglobulin and T cell receptor gene studies are now being employed to diagnose "borderline" lymphoma cases and to "geno-phenotype" between B and T cell malignancies. These studies are used also in leukaemia patients and indeed a recent report indicated that this technique is able to detect a single leukaemic cell among 500 normal marrow cells.

In conclusion I have attempted to summarise how molecular biology and the relevant techniques are currently changing the face of diagnostic and clinical haematology. This new DNA technology is no longer an event of the future. To quote Edward Beniz of Yale Medical School:

"The era when a Physician will require a working knowledge of the discipline of molecular biology and an ability to apply its power is no longer approaching; it is here".

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