INTERNAL MEDICINE GRAND ROUNDS

DNA IN MEDICINE: MOLECULAR GENETICS IN CLINICAL IMMUNOLOGY

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1. Glossary

Alleles

Variant forms resulting from mutations at a given genetic locus.

Anonymous probes

Probes for genetic loci of unknown function which are useful because they define a polymorphism that is in linkage with a mutation causing a specific disease.

Association

Increased prevalence of a genetic marker in unrelated individuals that carry a given trait or disease. Determined by population studies.

cDNA probes

Complementary DNA made from mRNA templates. cDNA can be inserted into plasmids, replicated in bacteria and selected for the desired genes.

Centimorgan (cM)

Distance between genetic loci determined by the frequency of crossing over. Genes that recombine once in every 100 meiotic opportunities are said to be one centimorgan apart. However, certain segments of DNA carry recombinational hot spots and are predisposed to crossing-over more frequently than would be predicted from their DNA length.

Cloning

Separation and independent propagation of a specific element from a population of similar elements, such as cells, antibodies or genes. To clone DNA fragments they are introduced into a simple, rapidly dividing organism in which the inserted piece replicates along with the host DNA.

Cosmid vector

Hybrid of plamid and phage used to insert DNA fragments into bacteria for cloning. Fairly large DNA fragments can be replicated in these vectors.

DNA hybridization

If two identical pieces of DNA are brought together, they will stick to each other. If only parts of them are identical the non-identical components will remain separate and can be easily removed.

DNA Sequencing

Determination of the nucleotides that comprise the DNA of a gene. From the DNA sequence the amino acid sequence of the protein it specifies can be deduced.

Gene cloning

Procedure used for obtaining the molecular probes with which DNA analysis is performed.

Gene conversion

Replacement of a segment of DNA with a fragment from another gene, resulting in an altered protein product.

Genes

Units of DNA that determine the structure of proteins.

Genetic code

Groups of three bases (codons) specify the amino acids to be incorporated into a protein.

Genomic DNA

Unmodified genetic material of an organism.

In situ hybridization

Detection of DNA binding to tissue, cells or chromosomes using appropriate histologic methods.

Kilobase (Kb)

Fragment of DNA 1000 base pairs long.

Libraries

A DNA library is constructed from genomic DNA or from complementary DNA (cDNA) made by the viral enzyme reverse transcriptase, from mRNA of a cell line or organ that synthesizes the gene product of interest. It is used for gene cloning.

Linkage

Tendency of genes to be inherited together when they are physically close on a segment of DNA. Determined by family studies.

Locus

Specific position where a gene is mapped on a chromosome.

mRNA

Messenger RNA, attaches to ribosomes in the cytoplasm to translate the coding sequences to produce protein.

Mutations

Change in the nucleotide sequence of a gene.

Nick translation

Procedure used to incorporate a radioisotope into a DNA probe.

Northern blotting

Procedure in which a DNA sequence is hybridized to complementary RNA.

Nucleotides

Component units of nucleic acids (DNA or RNA), each made up of a base attached to a sugar (deoxyribose and ribose, respectively). The base is attached to the sugar ring that in turn is bonded to a phosphate group. Nucleic acid molecules are long chains formed from nucleotides linked together by bonds running from the phosphate group of one nucleotide to the sugar group of the adjacent nucleotide. The bases (adenine, guanine, cytosine, thymine (DNA), uracil (RNA)) form the genetic code.

Oligonucleotide probes

Short sequences of DNA, usually 12-20 nucleotides long, produced synthetically, which can be used to detect mutant alleles in genomic DNA.

Phage vector

Bacterial virus, most commonly lambda phage, can accomodate larger DNA inserts than plasmids.

Plasmid vector

Bacterial plasmids are composed of short circular DNA encoding few genes, often including one for antibiotic resistance. The circle can be opened by digestion with a restriction enzyme and a fragment of DNA can be inserted and will then be replicated in the bacterial hosts.

Rearrangements

Recombinations that occur during the maturation of B cells and T cells and that bring together variable and constant genes to create functional antibodies and T cell receptors.

Recombination

Crossing-over or exchange of genetic material between homologous chromosomes during meiosis.

Restriction endonucleases

Bacterial enzymes that cleave double-stranded DNA at specific recognition sequences of bases. Restriction sites are highly specific. Availability of these enzymes paved the way for restriction mapping, restriction fragment length polymorphism analysis and powerful new methods for sequencing DNA.

Restriction fragment length polymorphisms (RFLP)

The presence or absence of a polymorphic restriction site provides a marker whereby one may determine from which chromosome (maternal or paternal) a particular gene of interest is derived, and thereby provides a label with which to follow the inheritance of a specific chromosomal segment within a family.

Restriction sites

Specific nucleotide sequence that is susceptible to cleavage by a given restriction endonuclease. The fragments of DNA of different sizes generated, can be separated by agar gel elotrophoresis. Presence of a DNA sequence specific for a given gene can be detected with a radioactive probe.

Southern blotting

Procedure developed by E.M. Southern, which consists of cutting high molecular weight DNA with restriction enzymes, separating the fragments of different sizes by electrophoresis in agarose, transferring the DNA from the gel to an appropiate filter and hybridizing on the filter with a labeled probe. The resulting autoradiographs produce a precise and reproducible pattern of the DNA fragments that contain the gene.

Transcription

Production of mRNA complementary to the sequence of nucleotides in a DNA gene. The resulting pre-messenger RNA contains both coding and non-coding sequences. The latter are subsequently removed to produce mature mRNA.

Translation

Process by which a protein molecule is synthesized by bringing in amino acids in the proper order as encoded in the nucleotide sequence of the messenger.

tRNA

Transfer RNA, attaches to individual amino acids and brings them

into position next to the codons of the mRNA. Thus tRNA mediates the translation of the code and assures that the amino acids are assembled in the proper order.

Walking the genome

Or chromosome walking is used to analyze long stretches of DNA. In this technique a library is screened for overlapping segments.

Western blotting

Detection of protein antigens by binding of labeled antibody to protein on a nitrocellulose gel transfer.

2. Introduction

The new DNA technology offers tools for understanding, diagnosing and possibly treating a variety of diseases. Many of these are as expected, inborn errors of metabolism or Mendelian disorders such as abnormalities of hemoglobin. More surprising, perhaps, is the utilization of restriction fragment length polymorphisms (RFLP) for the localization of genes causing diseases in which the mechanism is entirely unknown, as in the case of cystic fibrosis, Huntington Disease, or muscular dystrophy. Also somewhat unexpected, are the applications of nucleic acid hybridization in the area of infectious diseases. Particularly interesting is the possibility of searching for the presence of viral nuclear materials in chronic infections which are usually not thought to be infectious in origin. In the area of immunology, DNA technology has had an important impact on the understanding and definition of HLA antigens and many new associations of diseases with RFLPs obtained with HLA probes are being described. Also fascinating are the new approaches to the study of polymorphisms associated with the immunoglobulin genes and the genes of the alpha and beta chins of the T-cell receptor for antigen. A glossary of terms used in this paper, and in the references on which it is based, has been provided to aid those who may not be familiar with the language of molecular genetics.

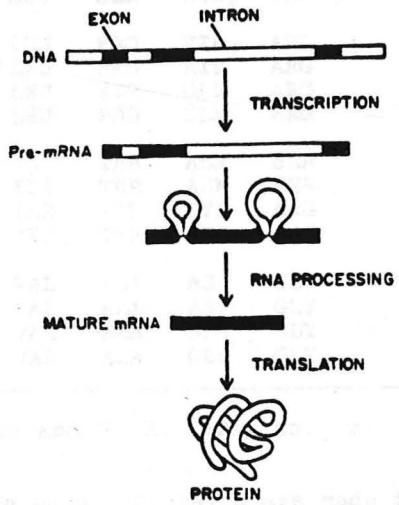

FIGURE 1. The entire gene, including both introns and exons, is transcribed into a pre-mRNA. Intron transcripts are then spliced out as the pre-mRNA is processde into mature RNA, which is translated on the ribosomes into protein. (From C. A. Francomano and H. H. Kazazian, Jr., Annual Review of Medicine, 1986.)

3. Working with DNA.

3.1. How genes work. Genes are stretches of DNA that contain the coding sequences or exons, as well as intervening spaces, called introns, which are transcribed to provide the template for the synthesis of proteins. This mechanism exists in all forms of life and governs the insertion of amino acids into polypeptide chains.

3.2. The genetic code. The genetic code consists of sets of three nucleotides, or codons, which spell out which amino acids are to be used in putting together the polypeptide chains.

Table 1 The genetic code

Nucleotide 1	Nucleotide 2				Nucleotide 3
	T	C	A	G	
T	PHE	SER	TYR	CYS	T
	PHE	SER	TYR	CYS	C
	LEU	SER	Stop	Stop	A
	LEU	SER	Stop	TRP	G
C	LEU	PRO	HIS	ARG	T
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLN	ARG	A
	LEU	PRO	GLN	ARG	G
A	ILE	THR	ASN	SER	T
	ILE	THR	ASN	SER	C
	ILE	THR	LYS	ARG	A
	MET	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	T
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

(From C. A. Francomano and H. H. Kazazian, Jr., Annual Review of Medicine, 1986.)

3.3. How proteins are made. Proteins are made in the cytoplasm, at the ribosomes, where messenger RNA interacts with specific transfer RNAs which bring the correct amino acids into an orderly sequence.

3.4. Restriction enzymes. Much of what one can do with DNA today is based on the discovery of bacterial enzymes, called restriction endonucleases, which cleave DNA at specific sites. The discovery of restriction enzymes was responsible in large

part for the rapid progress of molecular genetics. Some of the commonly used restriction enzymes are shown in Table 2.

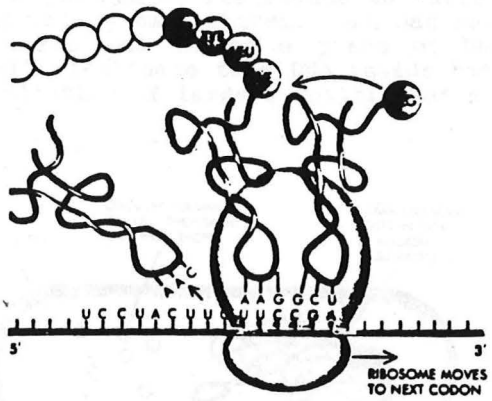

FIGURE 2. At the ribosome, the codons of a messenger RNA molecule base-pair with the anticodons of transfer RNAs, which are charged with amino acids. (From J. D. Watson, J. Tooze, D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

Table 2 Some restriction enzymes and their cleavage sequences

Microorganism	Abbreviation	Sequence (5'——3' / 3'——5')
Bacillus amyloliquefaciens H	*Bam*HI	↓ GGATCC / CCTAGG ↑
Haemophilus aegyptius	*Hae*III	↓ GGCC / CCGG ↑
Haemophilus influenza Rd	*Hind*III	↓ AAGCTT / TTCGAA ↑
Haemophilus parainfluenzae	*Hpa*I	↓ GTTAAC / CAATTG ↑
Providencia stuartii 164	*Pst*I	↓ CTGCAG / GACGTC ↑

(From C. A. Francomano & H. H. Kazazian, Jr., Annual Review of Medicine, 1986.)

3.5. Plasmids. These are small DNA molecules of bacteria that contain genes which convey resistance to antibiotics. They are very simple and easily manipulated. One can snip them open using restriction enzymes and insert a piece of DNA one wishes to study. They will replicate this DNA inside bacteria and offer a factory for production of large quantities of a given DNA.

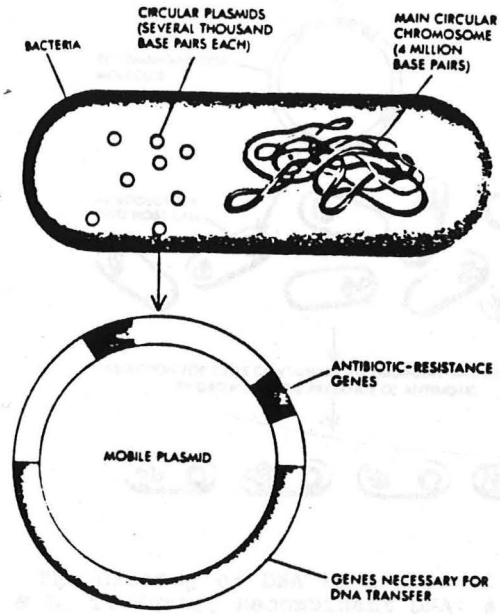

FIGURE 3. Plasmids are small, autonomously replicating bacterial DNA molecules containing genes conveying resistance to specific antibiotics. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

3.6. Cloning of DNA in a plasmid. As shown in Figure 4, plasmids containing our DNA sample and an antibiotic resistance marker-gene, allow selection of cells containing the recombinant DNA molecules by growth of the bacteria in presence of the antibiotic. The desired colonies containing the genes we wish to select can then be identified, either by hybridization with a radioactively labeled DNA probe, as shown in Figure 5, or if the protein product is made in the bacteria, by using a specific antibody that recognizes the protein encoded by the gene that is being cloned.

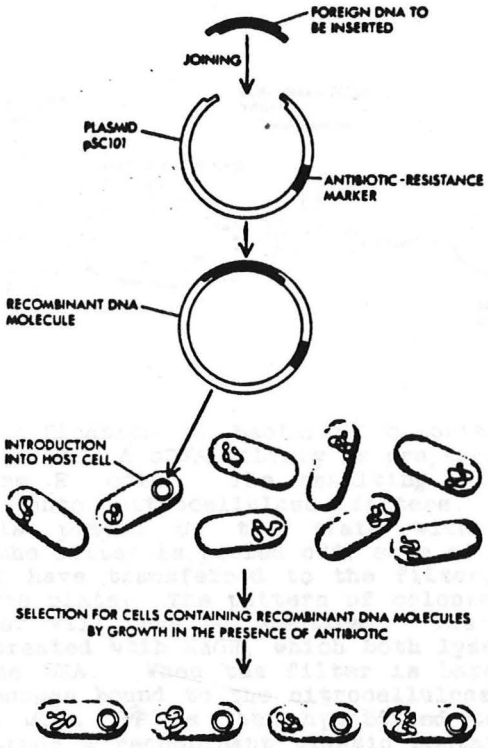

FIGURE 4. The cloning of DNA in a plasmid. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

3.7. Expression of cloned genes.

3.8. Southern hybridization. One of the techniques most widely used in DNA studies is that of Southern blotting. This technique, which is named after Dr. E. M. Southern, is described in Figure 7. A variation of this technique, which has been called Northern blotting, consists of hybridization of DNA to RNA. At this point it may be wise also mention Western blotting, a procedure in which proteins are blotted onto nitrocellulose and the bands are developed by autoradiography, after their reaction with specific antibodies labeled with a radioactive tracer.

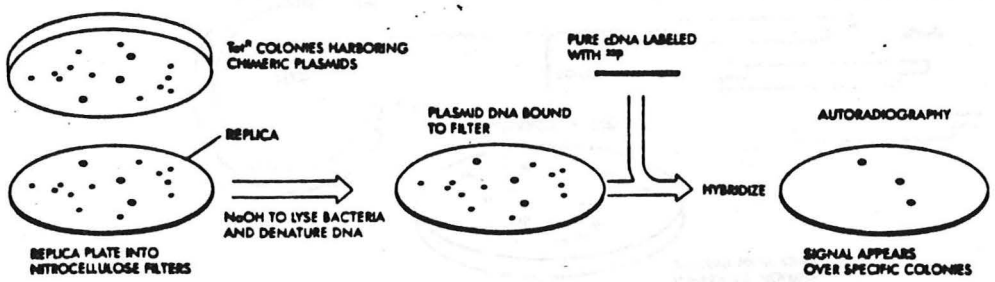

FIGURE 8. Identification of bacterial colonies harboring a specific cDNA plasmid. A cDNA library is prepared in pBR322 and used to transform E. coli. The resulting colonies can be "replica-plated" onto nitrocellulose filters. A sheet of nitrocellulose is placed on the plate with the bacterial colonies. When the filter is peeled off, some of the bacteria in each colony will have transferred to the filter, and the rest will remain on the plate. The pattern of colonies on the plate and on the filter will thus be identical. The nitrocellulose filter is then treated wtih NaOH, which both lyses the bacteria and denatures the DNA. When the filter is baked in a vacuum oven, the DNA becomes bound to the nitrocellulose. A pure cDNA or mRNA labeled with ^{32}P is then hybridized to the filter. Bacteria that harbor a recombinant plasmid containing sequences homolougous to the labeled probe will hybridize to it, and give a signal following autoradiography. The position of the positive colony on the replica filter can be compared with the master plate, and that bacterial colony can be picked and expanded. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

3.9. **Restriction fragment length polymorphisms.** Analysis of RFLPs is a procedure which is based on the fact that if restriction sites are modified through mutations, genes detected with specific DNA probes will reside on fragments of different sizes. An example showing how this method of investigation is applied in the study of sickle cell hemoglobulin mutation is shown in Figure 8.

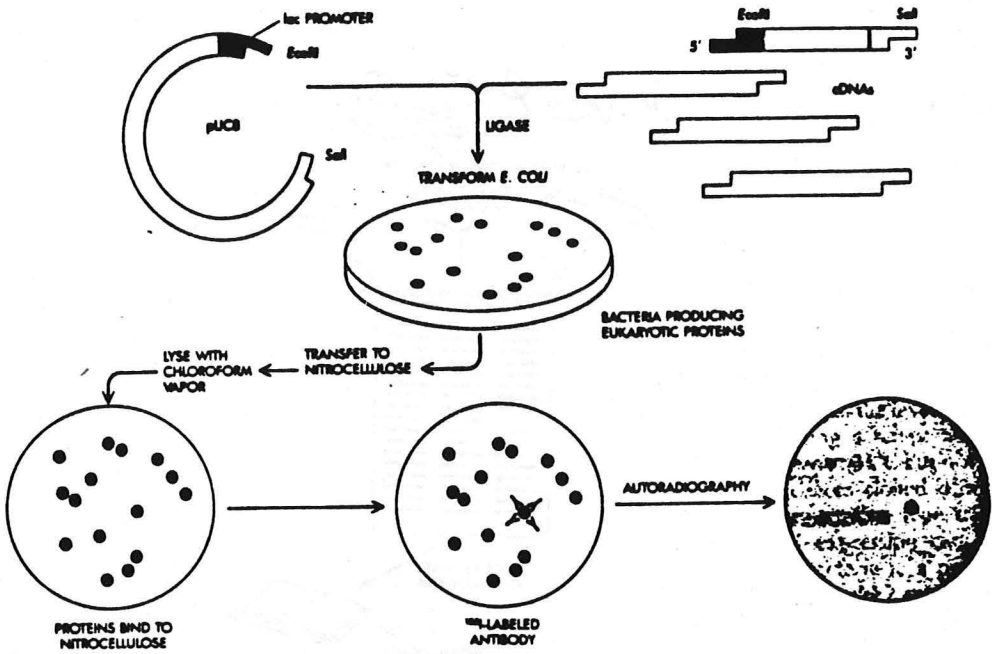

FIGURE 6. The ability to express eukaryotic proteins in E. coli can be used to identify and isolate specific eukaryotic cDNA clones. The cDNA for chicken tropomyosin was cloned using this method. A cDNA library was made from chicken smooth-muscle mRNA. (Tropomyosin represents about 0.5 percent of smooth-muscle mRNA.) The cDNA was cloned into pUC8 next to the lac promoter in such a way as to ensure that all the cDNAs were in the correct orientation relative to the direction of transcription from the promoter. All the resulting bacterial colonies should have produced one eukaryotic protein. Colonies that produced tropomyosin (and that therefore had the tropomyosin cDNA clone) were identified by screening the library, essentially as described in Figure 5, except that a labeled antitropomyosin antibody was used rather than a labeled cDNA. Colonies producing tropomyosin bound the labeled antibody and were identified by autoradiography. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

3.10. Oligonucleotide probes. When a mutation modifies a gene without causing major changes in restriction sites, RFLP analysis is not practical. However, if the sequence of nucleotides involved in the mutation is known, it is possible to synthesize small probes in the laboratory and as shown in Figure 9, to devise conditions under which such a probe will bind to the normal gene and not to the mutant.

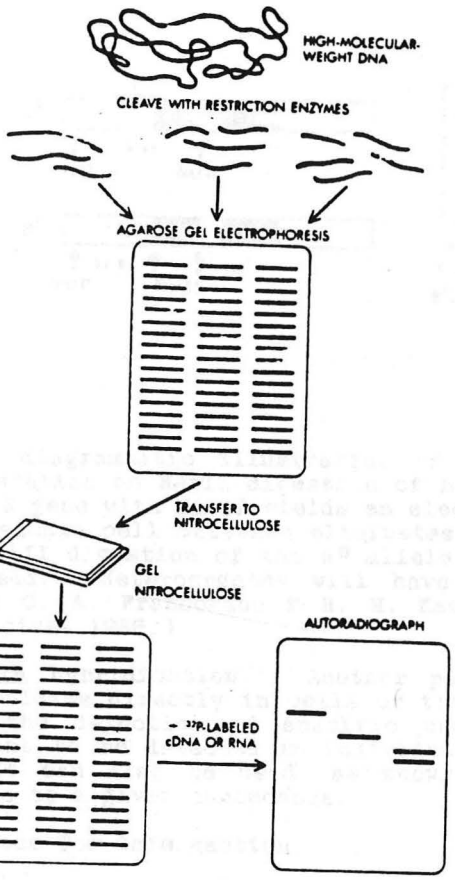

FIGURE 7. Southern blotting. Eukaryotic DNA is cleaved with one or several restriction enzymes. The cleaved DNA is separated by size using agarose gel electrophoresis. The gel is then laid on a piece of nitrocellulose, and a flow of buffer is set up through the gel onto the nitrocellulose. This causes the DNA fragments to flow out of the gel and bind to the filter. A replica of the DNA fragments in the gel is created on the filter. The filter can be hybridized to a suitable labeled probe, and specific DNA fragments that hybridize to the probe will give a signal following autoradiography. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course.)

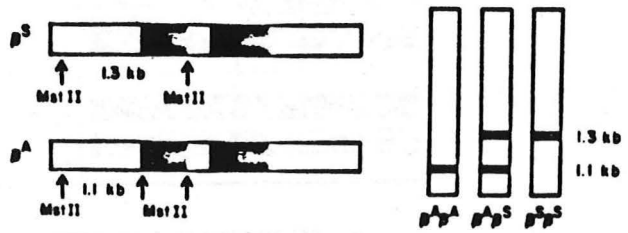

FIGURE 8. A diagrammatic illustration of the effect of the sickle cell mutation on MstII digestion of human DNA. Cleavage of the normal β gene with MstII yields an electrophoretic band at 1.1 kb. The sickle cell mutation eliminates one specific MstII site and so MstII digestion of the β^S allele produces a 1.3 kb-fragment instead. Heterozygotes will have fragments of both sizes. (From C. A. Francomano & H. H. Kazazian, Jr., Annual Review in Medicine, 1986.)

3.11. **In situ hybridization.** Another powerful tool is the ability to hybridize directly in cells or tissues. This allows, for example, the detection of specific nucleic acids of an infectious agent to be detected in individuals having a chronic infection. It can also be used, as shown in Figure 10, to localize a gene to a given chromosome.

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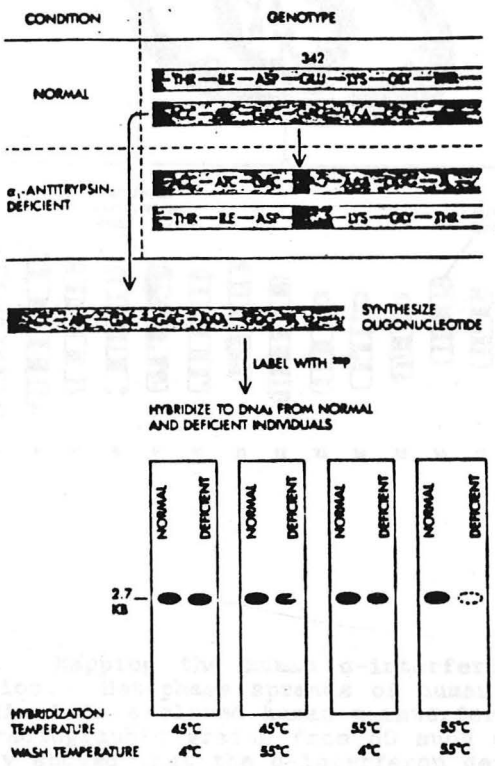

FIGURE 9. Diagnosis of α_1-antitrypsin deficiency by using a synthetic oligonucleotide. DNA from a patient with α_1-antitrypsin deficiency was analyzed and found to have a single base change (G → A) in the protein-coding region. A 19-base oligonucleotide was synthesized chemically to be complementary to the normal gene (and therefore to have one mismatch with the mutant gene). This oligonucleotide can distinguish between the normal and mutant genes when it is used as a probe in Southern blot analysis. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A Short Course, 1983.)

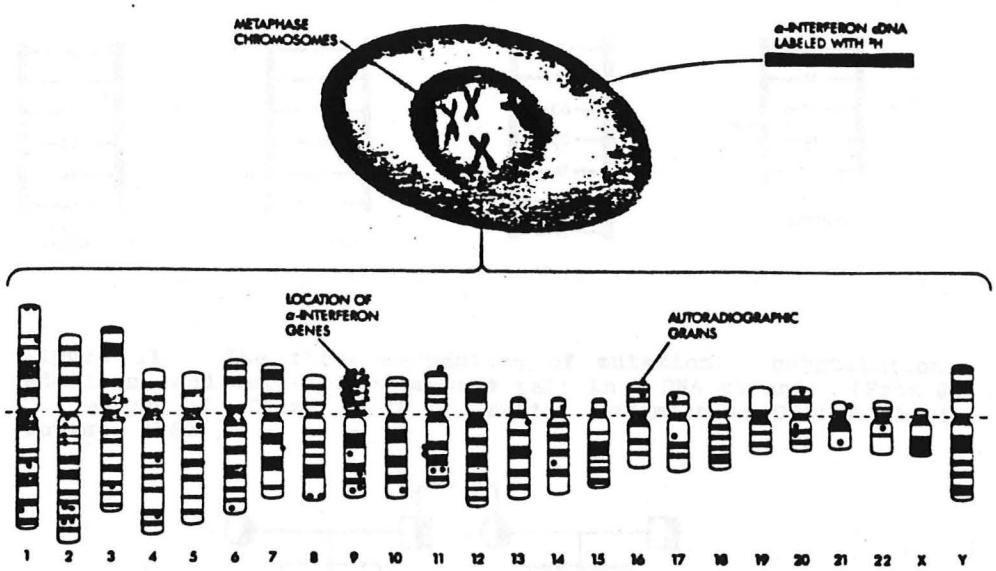

FIGURE 10. Mapping the human α-interferon gene by in situ hybridization. Metaphase spreads of human cells were prepared and hybridized to a cloned human α-interferon cDNA labeled with ^{3}H. Autoradiographic grains from 50 such spreads were totaled and clearly showed that the α-interferon genes are on chromosome 9. (From J. D. Watson, J. Tooze, & D. T. Kurtz, Recombinant DNA: A short Course, 1983.)

4. DNA in diagnosis of genetic diseases.

DNA techniques are now being used (1) to precisely define mutations in diseases in which the mutant gene is known, (2) to elucidate genetic defects in conditions where the mutant gene is unknown and (3) for the prenatal diagnosis of disease or pre-symptomatic diagnosis of individuals at risk. There are three mechanisms by which mutations occur (Figure 11). They are: substitution, addition, or deletion of nucleotides from the coding sequence of a gene. Such defects may occur at just one location (point mutations) or may represent gross alterations, such as insertions, deletions, and rearrangements of larger stretches of DNA. The example shown in Figure 12, illustrates a family in which a gene deletion occurred. The affected individuals had growth hormone deficiency.

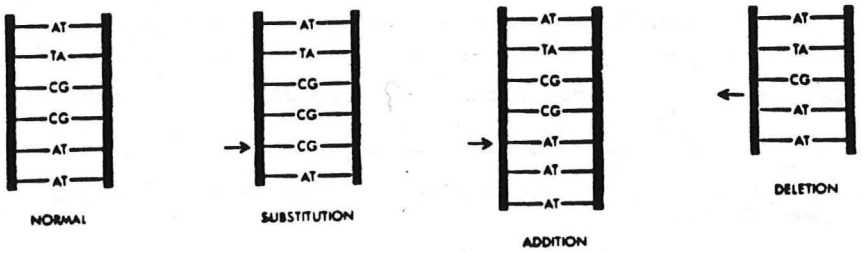

Figure 11. The three mechanisms of mutation: substitution, addition, and deletion of a base pair in a DNA strand. (From J. D. Watson, J. Tooze, & D. T. Kurtz; Recombinant DNA: A Short Course, 1983.)

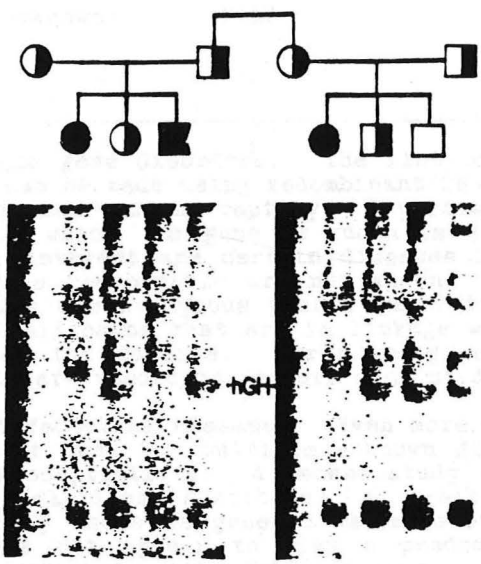

FIGURE 12. This Southern blot illustrates a gene deletion in a family with human growth hormone deficiency. Children homozygous for growth hormone deficiency in this family lack the band representing human growth hormone (hGH) gene sequences on these blots. The individuals in the pedigree represented by blackened symbols are affected and lack the hGH gene, while individuals represented by the half-blackened squares and circles are heterozygous males and females, respectively. In this family, the disease-producing mutation is a deletion of the growth hormone gene. (From C. A. Francomano & H. H. Kazazian, Jr., Annual Review of Medicine, 1986.)

Table 3 DNA in diagnosis of genetic diseases

Genetic Locus	Mutation	Technique	Examples
Known	Deletion	gene probe	growth hormone deficiency osteogenesis imperfecta
	Substitution	oligonucleotide probe	α_1-antitrypsin deficiency sickle cell hemoglobin
	Unknown	RFLP	phenylketonuria β-thalasemia ornithine transcarbamylase deficiency
Unknown	Unknown	RFLP	Huntington disease adult polycystic disease Wilson disease cystic fibrosis

4.1. Single gene disorders. The list of diseases in which a diagnosis can be made using recombinant DNA technology is already quite large and growing rapidly. A partial list of single gene diseases in which the gene is known is given in Table 4. Of particular interest are certain diseases in which the mechanism and the gene responsible are not known. The probes utilizing such studies are anonymous probes which define polymorphisms of unknown significance that are in linkage with the (unknown) gene that causes the disease. Large pedigrees with many affected individuals are needed to perform such studies.

4.2. Multifactorial Diseases. Even more difficult are diseases in which not one, but multiple unknown genetic loci combine to generate susceptibility. A recent study on affective disorders in Amish families suscitated a great deal of interest. Unfortunately there is genetic heterogeneity, so that the same factor does not appear to play a predominant role in other families affected with this disease. Other multifactorial diseases that are being investigated with DNA probes (Table 6) are atherosclerosis and certain types of cancer.

4.3. Prenatal diagnosis by DNA analysis. In the absence of successful therapy, and since these diseases cause much suffering, efforts have gone into early diagnosis of affected fetuses to offer abortion. The techniques of recombinant DNA offer the possibility of diagnosing the mutations directly, instead of having to assay for a product. Some of the conditions in which prenatal diagnosis, using DNA analysis is currently available are shown in Table 7.

Table 4 Single gene diseases - gene known

Alpha-1-antitrypsin deficiency
Familial amyloidotic polyneuropathy
Familial hypercholesterolemia
Growth hormone deficiency
Hemophilia A and B
Hereditary angioedema type I (C1 inhibitor)
Lesch-Nyhan syndrome (HPRT deficiency)
Ornithine transcarbamylase deficiency
Osteogenesis imperfecta
Phenylketonuria
Sickle cell hemoglobin
Thalasemias

Table 5 Single gene diseases - gene not known

Adult-onset polycystic kidney disease
Alzheimer disease
Cystic fibrosis
Duchenne muscular dystrophy
Huntington disease
Wilson disease
X-linked chronic granulomatous disease

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markers and bipolar affective illness. Nature 326 289-292, 1987.

Table 6 Multifactorial diseases

Affective disorders
Linkage with DNA markers on chromosome 11 in Amish
Belgian study showed linkage with markers on chr 10
Atherosclerosis
Apolipoprotein AI and AII
Apolipoprotein B
Cancer
Retinoblastoma, linkage with loci on chr 13
Osteosarooma

Table 7 Prenatal Diagnosis by DNA Analysis

α_1-antitrypsin deficiency
Carbamyl phosphate synthetase deficiency
Duchenne muscular distrophy
Hemophilia A & B
Hypoxanthine-quanine phosphoribosyl transference deficiency
Ornithine transcarbamylase deficiency
Phenylketonuria
Sickle cell diseae
Thalasemias aplha & beta

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5. DNA hybridization for diagnosis of infections.

With the development of molecular probes for genetic material of infectious agents, investigators are beginning to utilize the high-sensitivity and specificity of recombinant DNA techniques for the diagnosis of infectious diseases. This approach is particularly pertinent when applied to agents for which isolation, and characterization are tedious, difficult, or impossible.

Table 8 DNA hybridization for diagnosis of infections

Pathogen	Probe	Specimen Examined
E. coli producing Enterotoxin	heat stable/unstable toxin genes	stool isolates stool specimens contaminated food isolates
Gonococcus	gonococcus cryptic plasmid	swabs of male urethra
Cytomegalovirus	selected viral fragments	urine white blood cells lung tissue
Epstein-Barr virus	Viral DNA	infected cell lines
Herpes simplex virus	thymidikinase gene and other viral DNA	viral isolates
Adenovirus	viral DNA	swabs of nasopharynx stool specimens
Hepatitis B virus	viral DNA	liver serum
Plasmodium falciparum	oligonucleotide	blood
HIV	viral DNA	infected cells

5.1. Bacterial, viral and parasitic diseases. Some examples of diseases caused by infectious agents in which this technology is currently being applied are shown in Table 8. Particularly interesting is the application of these techniques directly to the study of tissue biopsies, in conditions where the role of infectious agents is uncertain. For example, Coxsackie B viral

nucleic acids have been found in muscle biopsies of some patients with polymyositis and Epstein-Barr virus has been demonstrated to be present in salivary glands of patients with Sjogren syndrome. On the other hand, the investigation of synovial membranes of patients with rheumatoid arthritis failed to disclose presence of nucleic acid of Epstein-Barr virus. In other studies, a single oliglonucleotide probe has been found to be useful for the diagnosis of plasmodium falciparum in droplets of blood collected on filter papers from many parts of the world.

5.2. Detection of viral nucleic acids in hosts with chronic infectious and noninfectious diseases. Examples of the application of recombinant DNA techniques for the investigation of viral agents in some chronic conditions are shown in Table 9.

Table 9 Detection of viral nucleic acids in hosts with chronic infectious or noninfectious diseases

Disease	Probe
Chronic hepatitis	Hepatitis B virus
Latent CNS herpes simplex	Herpes simplex virus DNA
Latent varicella-zoster	Varicella-Zoster virus DNA
Acquired immunodeficiency	Human immunodeficiency virus
Nasopharingeal carcinoma and Burkitts lymphoma	Cloned EBV DNA
CNS lymphoma	Cloned EBV DNA
Hepato-cellular carcinoma in alcoholic liver diseases	Hepatitis B virus DNA

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6. DNA typing for HLA antigens.

The main histocompatibility complex is one of the most intensively studied genetic regions. Figure 12, shows a diagram of the mapping of the various genes on the short arm of chromosome number 6. The loci encoding the class I HLA antigens are located distally and include the genes for HLA-A,B,C antigens. They are followed by the class III, region which includes the loci for some complement components and 21-hydroxylase genes. They will be discussed below. The region of the class II antigens has been expanded to allow for the display of the many loci now known to exist in this region. This class II area includes the subregions for DR, DQ, DO, and DP antigens.

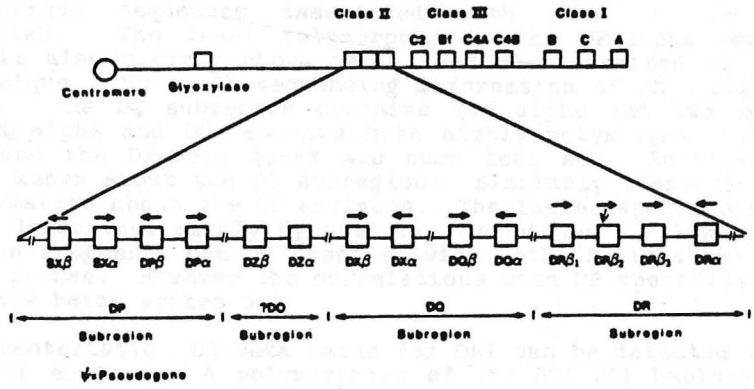

FIGURE 13. Mmap of Class II MHC genes on the short arm of chromosome 6 (taken from reference 30). The exact orientation of individual genes with respect to each other is not exactly known, although the direction of transcription is indicated by the arrows. The location of the DO subregion is not known and may be telomeric to the DR subregion rather than between the DP and DQ subregions as shown in this diagram.

6.1. Class I antigens. Southern blotting with probes for class I antigens, of DNA digested with a variety of enzymes has produced a large number of bands varying from 15 to 25. This finding is compatible with the presence of a large number of class I genes. Analysis of the distribution of polymorphic bands has revealed many that appear to correlate with HLA-A locus specificities and fewer that appear to be associated with HLA-B locus antigens.

6.2. Class II HLA antigens. Much more information exists about restriction fragment length polymorphisms that are associated with the class II antigens. The availability of B-cell lines derived from individuals that are homozygous by descent has facilitated efforts to establish patterns associated with haplotypes and their correlation with serologic specificities. Studies of the HLA class II antigens have also utilized information from functional studies with allostimulated T-cells, from biochemical methods, such as two-dimensional polyacrylamide gel electrophoresis, and from DNA sequencing of the genes. For the purpose of this discussion we will briefly review information about each of the class II subregions and touch upon some salient features of the major haplotypes.

The DR subregion contains one alpha gene and one to three beta genes. In many haplotypes that have three DR-beta genes, the second one is a pseudogene and is not expressed. Of the genes in the DR subregion, DR-beta-1 is usually the most polymorphic. It carries unique sequences associated with each of the DR specificities. The least polymorphic is the DR-alpha gene. However, it also clearly shows variations when examined by the RFLP technique. Not much sequencing information of DR-alpha is available. The DQ subregion contains two alpha and two beta genes. DQ-alpha and DQ-beta are both highly polymorphic. The DX-alpha and the DX-beta genes are much less so. Relatively little is known about the DO subregion. Similarly there is not much information about the DP antigens. The latter specificities are still determined mainly by cellular techniques. Polymorphic restriction fragments can be observed with both DP-alpha and DP-beta gene probes. However the correlations with DP specificities are only now being worked out.

DR1. Characteristic DR-beta bands for DR1 can be detected with a number of enzymes. A polymorphism of the HLA-DR1 haplotype, characteristic of an Israeli population, and detected by cellular, serologic, as well as DNA techniques, has recently been described by Cohen and co-workers. Another specificity, called DR-BON by French workers, and DR 'BR' in England, has recently been described. Cells carrying this haplotype can be distinguished by cellular techniques. They react with some but not all DR1 sera. At the genomic level, they have been indistinguishable from DR1. Probing with DQ-beta and several different restriction enzymes shows bands that correlate with DR1 and which are distinct from the DQ-beta bands associated with

DR2. Thus the DQw1 specificity, defined by serology, can be easily subdivided by RFLP.

DR2. This specificity formerly believed to be rather simple, can now be split into several distinct haplotypes. In Table 10, are shown four major types that can be differentiated with homozygous typing cells. While some of the information about allelic forms in the different loci is somewhat tentative at present, the complexity of DR2 is readily apparent. Particularly interesting is the DR2-short haplotype typed as DB9 with homozygous typing cells, which carries DQw3 instead of the usual DQw1.

Table 10 Complexity of HLA-DR2

Serology	HTC	DPβ	DPα	DQβ	DQα	$DR_{\beta 3}$	$DR_{\beta 2}$	$DR_{\beta 1}$	DRα
DR2 long	Dw2	w4	w4	2,6	2	2	-	w2	2
DR2 long	Dw12	w4	w4	12	2	2	-	w12	2
DR2 short	AZH	w3	w3	1	2	2s	2s	2s	2
DR2 short	DB9	w2	w2	w3	4	2s	2s	2s	2

Table 11 HLA-DRw6

DRw13	Dw18	DQw1
	Dw19	DQw1
	-	DQw3 TA10-positive
DRw14	Dw9	DQw1
	Dw16	DQw3 TA10-positive

Table 12 Type I Diabetes is associated with TA10 - negative DR4,DQw3 haplotype

HLA-DR4, DQw3	Diabetes Patients N	%	Control Subjects N	%
TA10-Positive	4	7.3	11	55.0
TA10-Negative	51	97.7	9	45.0

RR = 10.4 $p<0.001$

DR3. Several subsets of DR3 haplotypes can be defined by RFLP with the DR-beta probe. In addition to differences in DR-beta-1, the DR-beta-3 gene associated with the DR3 haplotypes has recently been shown to be expressed in two allelic forms. These

appear to correlate with the LBQ1 specificity defined by T cells. When examined with the DQ-beta probe, most of the cell lines had identical patterns with the exception of one which was distinct in the studies by MacMurray and co-workers.

DR4. The complexity of DR4 is well-known. A number of different types can be defined by cellular techniques including Dw4, Dw10, Dw13, Dw14, Dw15, KT2, and KT3. Compared with this florid subtyping, the RFLP patterns with DR-beta have been relatively monotonous, although some polymorphisms with DR-beta among homozygous DR4 cell lines have been recently described. Gregersen and co-workers, have shown by DNA sequencing, that the DR4 subtypes can be distinquished on the basis of one or two amino acid substitutions on the DR-beta-1 chain. A major difference is the presence of the DQ-specificity Wa (instead of DQw3) associated with DR4,Dw15 which is of Japanese origin.

DR7. The DR-beta-3 gene of the DR4 haplotype appears to be the same as that of the DR4 cells. The relationship between DRw52 and DRw53 has recently been discussed by Gorski and co-workers, and Figure 13, is taken from their paper. DR7 includes three major subsets defined by T-cell typing: Dw7, Dw17, and Dw11. The Dw11 haplotype is distinct in that its DQ component is DQw3 rather than DQw2. These haplotypes can be readily distinguished by RFLP.

DRw6. As shown in Table 11, DRw6 is split serologically into DRw13 and DRw14. By cellular techniques, it is subdivided into Dw18, Dw19, Dw9, and Dw16. DRw6 cells either of the 13 or 14 Dw16 type that carry DQw3, rather than DQw1, are usually TA10-positive. It is now possible to define these subsets by RFLP with DR-beta and DQ-beta probes.

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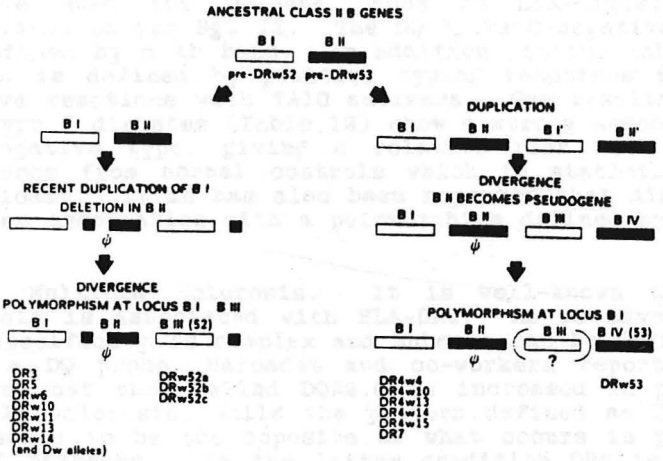

FIGURE 14. Schematic representation of the evolutionary tree of the DR_β chain gene family. A two-locus family is postulated as the mammalian ancestral arrangement. The DRw52 lineage (left) has undergone a deletion of most of the ancestral $DR_\beta II$ locus. The $DR_\beta I$ locus and promoter region of the $DR_\beta II$ locus were duplicated. These two events could have happened in opposite order or simultaneously. In the DRw53 lineage the ancestral $DR_\beta I$-$DR_\beta II$ pair was duplicated. Whether locus $DR_\beta III$ has been deleted or maintained will only be known when the linkage maps are avilable. Polymorphic variations were introduced at the $DR_\beta I$ loci at a higher rate than at the other loci, thus establishing haplotypic ($DR_\beta I$) and supertypic (DRw52 and DRw53) loci. (From J. Gorski, P. Rollini, and B. Mach, Immunogenetics, 1987.)

7. Disease associations with HLA RFLPs. As soon as the RFLP technology became available, studies were initiated in various diseases, in the hope of identifying unique bands. It was speculated that such bands might represent subsets of HLA antigens as yet not identifiable by serology, or they might signal the presence of other markers that might be associated with disease susceptibility more intensely that the HLA antigens themselves. Some of the reported findings will be reviewed briefly below.

7.1. Diabetes Type I. Several workers have reported on RFLPs that appear to subdivde the DQw3 antigen. Bands associated with the TA10 specificity were found to be markedly decreased in DR4-positive patients with diabetes mellitus type I, using a DQ-beta probe and several different restriction enzymes. It is possible to show that an allelic form of this specificity, defined as DQw3, TA10-negative, can be readily identified. In our own work we have used the DQ-beta probe on DNA-digested with the restriction enzyme Bgl II. The DQw3, TA10-negative specificity was defined by 6 kb band. In addition, in our laboratory this antigen is defined by positive typing reactions for DQw3 and negative reactions with TA10 antisera. Our results in patients with Type I diabetes (Table 12) show a strong association with a TA10-negative type, giving a relative risk with 10.4 and a difference from normal controls which is statistically highly significant. It is has also been reported that diabetes type I shows an association with a polymorphism defined by the DX-alpha gene.

7.2. Multiple Sclerosis. It is well-known that multiple solerosis is associated with HLA-DR2. As we have seen above, this specificity is complex and subsets can be defined by RFLP. Using a DQ probe, Marcadet and co-workers reported that the pattern that they called DQR2,6 is increased in patients with multiple solerosis, while the pattern defined as DQR1, is not. This seems to be the opposite of what occurs in patients with type I diabetes. In the latter condition DR2 is known to be decreased, but when it is present, it usually gives a DQR1 type RFLP pattern.

7.3. Narcolepsy. There is a remarkable association of narcolepsy with HLA-DR2. Almost every patient with typical narcolepsy that has been typed has had this antigen. The relationship with RFLP has been examined in both in France and in Japan and in both populations when the DNA was analyzed with the DQ-beta probe, the pattern that Cohen and co-workers, called DQR2,6 was almost universally found among the narcolepsy patients.

7.4. Celiac disease. Celiac disease has previously been shown to be associated with DR3 and DR7, and has been stated to have a primary association with DQw2. Interestingly, Howell and co-workers, have recently reported that using a DQ-beta DNA probe and the restriction endonuclease RSA1, they have detected a

polymorphic 4.0 kb fragment, which in DQw2 individuals was associated with a forty-fold increased relative risk of developing celiac disease. It should be pointed out, however, that this is a single report and is based on 20 patients and 11 controls.

7.5. Rheumatoid Arthritis. It is well-known that rheumatoid arthritis is associated with DR4. Among the DR4 subsets our initial observations clearly showed an association with Dw4. Subsequently, it has been shown by others, that also Dw14 may be increased in RA patients whereas Dw15 is associated with RA in Japanese. Some of the other subsets of DR4 such as Dw10 or Dw13, do not appear to be associated with this disease. Nepom and co-workers, have developed oligonucleotide probes that permit the recognition of Dw4 and Dw14 by RFLP. They have reported that children as well as adults with factor-positive rheumatoid arthritis, who are homozygous for DR4, have increased frequencies of Dw4 and Dw14. A group from Alabama, has reported that a restriction fragment detected with Bam H1 digested DNA, probed with a DR-beta probe, showed a band observed more frequently in patients than in controls. Their report that this particular band is found also in DR4-negative RA patients, but not in DR4-negative controls, thus giving a higher relative risk, requires confirmation.

7.6. Myasthenia Gravis. Another preliminary report involves an observation of a DQ-beta RFLP patients with myasthenia gravis. Bell and co-workers, studied 16 patients who were DR3 and had myasthenia, with the enzyme Hinc2. A 15 kb restriction fragment was found in 7 of the 16 patients and in only one of 19 healthy DR3 controls. Interestingly, this fragment was not observed in 24 patients with diabetes, celiac disease or premature ovarian failure, all of which had DR3. These results suggest that DQw2 is being split and that an unusual subset of DQw2, perhaps similar to that seen in DR7 haplotypes, was found in DR3 patients with myasthenia.

7.7. Ankylosing spondylitis. The association of ankylosing spondylitis with B27 is well-known. However efforts to elucidate this very strong association by attempts to identify subsets of B27 that might be prevalent in patients and absent in controls have uniformly met with failure. Subsets of B27 have been described using T-cell clones, monoclonal antibodies, isoelectric focusing, and sequencing of the B27 proteins. However, none of this information appears to correlate with the presence or absence of ankylosing spondylitis. McDevitt and co-workers, have isolated B27 genes from a random patient and a random normal control and found them to be indistinguishable. Recently, MacDaniel and co-workers, have reported a restriction fragment length polymorphism that showed a strong association with ankylosing spondylitis. DNA was digested with PvuII and probed with an HLA-B7 cDNA probe. A 9.2 kb band was found in 36 of 53 patients and in 25 of 107 controls. Preliminary results from these workers and from another laboratory suggest that the fragment observed is not detecting a B locus gene and that it's

segregation in families is independent of HLA-B27. Thus the true nature of this finding is at the present time obscure.

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8. Other genes in the HLA region.

8.1. Complement components. cDNA probes for C2, factor B, and C4 have been isolated. Their loci have been studied with restriction enzymes and have revealed more genetic heterogeneity than that exhibited on analysis of the respective protein polymorphisms. These genes are located between the HLA-DR and HLA-B loci. The order of the loci has been established to be C2, BF, C4A, 21-hydroxylase A, C4B, 21-hydroxylase B. C4A 21-hydroxylase and C4B 21-hydroxylase B appear to represent duplicated genes. Complement component typing by DNA hybridization appears to offer a useful approach to define the haplotypes. DNA studies will undoubtedly be useful in pinpointing the location of defects in patients with complement deficiencies. In some cases, this information may simply supplement that obtainable by HLA typing procedures. Moreover, in diseases associated with complement deficiencies, such as the lupus-like syndromes observed in these subjects, DNA studies may yield more information than that can be obtainable by performing studies on the complement proteins.

8.2. 21-hydroxylase. Congenital adrenal hyperplasia results from an inherited defect in any of 5 enzymatic steps required to synthesize cortisol from chloresterol. These conditions have been reviewed in a recent paper by White and co-workers. It has been demonstrated that the HLA-link defect in 21-hydroxylase deficiency involves a structural gene for the corresponding cytochrome P450. A bovine cDNA clone was isolated that encoded part of the this enzyme and was used to study DNA samples from normal persons. There are two genes encoding 21-hydroxylase. They are called A and B and they are located respectively immediately adjacent to C4A and C4B genes. An HLA3,Bw47,DR7 haplotype, which is extremely rare in the normal population, comprises about 20% of the alleles in classic 21-hydroxylase deficiency is also seen with HLA-B14,DR1 and with HLA-A1,B8,DR3 haplotypes. It is now possible to provide prenatal diagnosis for parents who already have one child with 21-hydroxylase deficiency. Samples of corionic villus obtained at the end of 10-weeks gestation can be used for DNA extraction and subjected to Southern blot hybridization. In addition to using the 21-hydroxylase cDNA probe it is wise to also test for class I and class II genes.

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White-P-C. New-M-I. Dupont-B. Congenital adrenal hyperplasia (2). N-Engl-J-Med 316:1580-1586, 1987.

9. Immunoglobulin genes and T-cell receptor genes. The diversity of antibodies, a mystery for a very long time, is now understood as a consequence of the splicing of variable and constant genetic components of the immunoglobulin genes. Similar events take place in the evolution of T lymphocytes for the generation of their antigen-specific receptors. Thus, it is possible to identify percursor cells, in which the DNA that hybridizes with specific probes for immunoglobulin genes or T-cell receptor genes has a genomic or unaltered arrangement, from cells that have differentiated in the course of maturation in the immune system, in which these genes have rearranged. Each cell makes one antibody and therefore has one unique pattern of gene rearrangement. In a clone of such cells, or in a malignancy derived predominantly from one cell type, sufficient quantity of DNA, all rearranged in the same pattern can be obtained, so that the rearranged bands would be clearly visible on Southern blot hybridization. If, on the other hand, a polyclonal proliferation of cells is sampled, the DNA obtained would represent a mixture of many different kinds of rearranged patterns and the resulting blot would be a smear. It is this principle that has been applied to the diagnosis of clonality of lymphocyte malignancies by the technique of DNA hybridization. There is a now large literature describing the use of this technique for the diagnosis of clonality in B cell and T cell tumors.

Another application of restriction fragment length polymorphism analysis with immunoglobulin and T-cell receptor gene probes is the detection of allelic polymorphisms. This approach to the study of alleles is at this time still in its infancy. However, alleles of the heavy and light chains of immunoglobulins have been studied by serologic techniques for a long time. Multiple DNA fragment polymorphisms associated with immunoglobulin heavy chain genes have recently been reported. They have been shown to segregate in linkage to each other and to Gm allotypes. A recent report by Kaiser and co-workers, describes susceptibility to multiple sclerosis associated with an immunoglobulin gamma-3 restriction fragment length polymorphism. A preliminary report of RFLP with an immunoglobulin heavy chain gene in patients with IgA nephropathy has also recently appeared.

Polymorphisms associated with the alpha and beta genes of the human T-cell receptor have also been recently described. The possibility that inherited alleles of the T-cell receptor may restrict the T-cell repertoire of MHC-restricted T-cell responses is a provocative possibility. In one report the T-cell repertoire in mice was found to be restricted in this manner.

Several preliminary reports have appeared suggesting that some human diseases may be associated with polymorphisms of the T-cell receptor beta gene. More work is needed to establish the true significance of these provocative preliminary observations.

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Conclusion. Recombinant DNA techniques are providing exciting new approaches in biology and applications medicine. These powerful tools are rapidly becoming available for the study of a

many human diseases. Particularly exciting are the discoveries of restriction fragment length polymorphisms linked to diseases, in which neither the gene responsible, nor the defect that causes the disease, are as yet known. This so-called "upside-down" technology will be fascinating to follow. The use of these tools in the diagnosis of infectious agents is turning up some interesting surprises. The rapid progress made in the HLA area has markedly expanded our view, particularly of the class II genes. We may be getting closer to what is really associated with susceptibility for some of the HLA-associated diseases. The detection of allelic markers close to the immunoglobulin and the T-cell receptor genes is still too new to accurately judge the potential impact on disease susceptibility studies. It makes sense, particularly in the case of T cells, that can see foreign antigens only when presented together with MHC determinants, that the T-cell receptor would also be a determining factor in the potential for making beneficial as well as harmfull immune responses. In this context the MHC molecules and the T cell receptors may well be the two interlocking components of the same puzzle of why some people develop autoimmune diseases.