MOLECULAR ANALYSIS OF GENETIC DISEASES: AN OVERVIEW FOR CLINICIANS

ALI A. JAVED, PhD, YING HUANG, MS,
and ALLAN T. BOMBARD, MD, FACOG, FACS, FACMG

ABSTRACT The identification of fetal genetic disease has, for the most part, relied on examination of an end product, such as analysis of factor VIII levels obtained from cord blood in fetuses at risk for hemophilia. Advances in molecular genetics have shifted our focus in prenatal diagnosis away from protein product analysis toward etiology, making new discoveries gleaned from the Human Genome Project relevant to clinicians.

This review discusses the basic principles involved in gene-based diagnosis, highlighting the complexities of current approaches to molecular diagnosis of fetal genetic disease. Given an understanding of both the theory and practice of genetic analysis, the review covers the fundamental principles of molecular biology (structure, function, packaging, and regulation) and discusses recombinant DNA techniques presently used for the analysis of mutations.

Clinical examples are presented to introduce the techniques most commonly employed in service laboratories: direct detection assays, where the specific mutation is recognized, and indirect detection assays, useful for the deduction of an inheritance pattern where the actual mutation or its gene is not known but may be closely linked to known DNA polymorphisms.

Key words: allele, DNA, hybridization, polymerase chain reaction, polymorphism, restriction fragment length polymorphism.

INTRODUCTION

Our present understanding of the molecular basis of genetic disorders is principally due to the recent clinical advances in recombinant deoxyribonucleic acid (DNA) techniques. The revolution in development and implementation is now almost two decades in the making. Most of the molecular genetic techniques now in clinical use were previously limited to laboratories involved in basic research; currently, however, they are routinely available in service laboratories performing DNA diagnostics. This transition itself—molecular technology from pure research to DNA service laboratories—is an extraordinary example of the impact of molecular genetics. As a result of these advances, it is imperative that physicians and other health care professionals who provide the clinical bridge between patient and laboratory possess a sound understanding of molecular diagnostic techniques.

The revolution in recombinant DNA technology has improved our understanding of simple mutations as causes of disease. The molecular basis of genetic disorders is as varied as clinical medicine itself. The molecular etiology of disorders may be fundamentally straightforward, such as in sickle cell disease, which is the best understood and the first disease whose mutation was established at the DNA level. On the other hand, molecular genetics has delineated a whole new class of disease where *anticipation* is involved; that is, the phenomenon of apparently increasing disease severity in successive generations. Addressing the etiology of the more complex disorders that involve anticipation (eg, fragile X syndrome, myotonic dystrophy, and Huntington's disease) is often challenging. One must consider such variables as paternal versus maternal transmission of the mutant allele and the existence of additional factors, as noted in the association of *Apo E4* allele with late-onset Alzheimer's disease.

Finally, the field of molecular genetics has been the motivating factor for approaches to medical practice. Molecular technologies have propelled diagnostic medicine from a predominantly clinical specialty—dysmorphology—where physical diagnosis is key to the direct evaluation of DNA and RNA and its impact on protein expression. In part, the Human Genome Project serves as a framework for the construction of these advances and holds the promise of unraveling the mystery of human genetics. Ultimately, the real achievements from this project will be the prevention and treatment of human genetic afflictions. None of this will be accomplished without the support of the medical community and the public; to do this, one must first appreciate the process of testing for diseases with a molecular basis.

This review provides an avenue to greater understanding of these exciting developments. The recombinant DNA techniques presently used for the analysis of mutations are briefly explained. We present clinical examples as an introduction to the techniques most commonly employed in service laboratories: *direct*

Division of Reproductive Genetics, Department of Obstetrics and Gynecology, Montefiore Medical Center, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York.

Address correspondence and reprint requests to Allan T. Bombard, MD, Dept of Ob/Gyn, Montefiore Medical Center, 1695 Eastchester Rd, Suite L4, Bronx, NY 10461.

detection assays, where the specific mutation is recognized, and *indirect detection assays*, useful for the deduction of an inheritance pattern where the actual mutation or its gene is not known but may be closely linked to known DNA polymorphisms.

Human Genome, Polymorphisms, and Mutations

The human genome is generally considered to consist of about 3×10^9 base pairs (bp) per haploid chromosomal complement, ie, nearly 6×10^9 bp per diploid cell, encoded within the 22 autosomes, the X and Y sex chromosomes, and the mitochondrial genome. Just a glance at the human population makes it evident that a wide range of phenotypic variation exists between various races, ethnic groups, and other isolated groups. The human population is truly heterogeneous. These variations comprise not only phenotypic, but also *genotypic* differences—differences at the DNA level. For example, the wide variation of genotypes within blood groups ABO, MNSs, Rh, and the various forms (*alleles*) of other erythrocyte proteins have long been established.

The molecular variations that result in protein differences are encoded at the DNA level and can be inherited. Variations such as these within normal genes and proteins (which are, parenthetically, not deleterious) are termed *polymorphisms*. In other words, polymorphisms represent two or more forms of functionally similar yet genetically distinct and structurally different forms of DNA. A specific chromosomal site (*locus*) is said to be polymorphic when there are two or more alleles with a frequency in the population of 1% or greater. Those permanent changes in the DNA sequence that result in a disordered (disease) state are known as *mutations* (although, in the strictest sense, *any* permanent change in DNA sequence may correctly be termed a mutation); a mutation not causing a change in functional properties is termed a *silent mutation* and is equivalent to a polymorphism. Polymorphisms have been immensely valuable as genetic markers for gene mapping by *linkage analysis* and are used to both trace and deduce different inherited forms of alleles/genes in family studies.

The primary causes of changes in DNA sequence are random error during DNA replication and the effects of environmental mutagens. The entire human genome is copied by replication during *each* cell division. It is surprising, therefore, that the end result of replication is relatively error free; in fact, virtually all (>99.9%) DNA replication errors are corrected by the "proofreading" property of DNA polymerases. Overall, the replication error is 10^{-10} per bp per cell division. Since the human diploid genome contains approximately 6×10^9 bp of DNA, new mutations introduced per cell division due to replication error are less than 1 new base pair mutation per cell division.

During an adult's lifetime, there are approximately 10^{15} cell divisions; thus, thousands of new mutations could conceivably occur at nearly every base pair level of the DNA sequence. Fortunately, most mutations occur in *somatic* cells and are not inherited, as they would be in *germ* cells (sperm or egg). Only germ line mutations are passed on from generation to generation and are the basis of inherited disorders. Somatic cell mutations, although not inherited, may cause disease (eg, cancer), depending on the type of mutation of a particular gene in a particular tissue. Understanding genetic polymorphism, mutation, and mutation rates helps us appreciate the existence of our molecular heterogeneity and allows us to examine the molecular approaches available to analyze the population variations.

Direct and Indirect Analysis Methods

The choice of using direct methods for mutation analysis is restricted by our knowledge of the existence of a mutation and its type. The use of direct methods is clearly limited to the analysis of mutations that have previously been documented. In almost all cases, the gene responsible for a particular abnormality has already been identified. Sickle cell disease is an excellent example of direct DNA testing success; a discreet mutation is recognized and may be easily tested for in patients at risk, with virtually a 100% rate of detection. Direct methods *cannot* be used for disorders for which the gene has not been identified; instead, linkage with polymorphic markers is used to deduce the inheritance pattern of a particular allele.

Until recently, linkage analysis was the sole technique for detecting most genetic disorders (eg, Huntington's disease, cystic fibrosis, and myotonic dystrophy). On identification of a particular gene and etiologic mutations, direct analysis for each mutation can be performed. For example, in cystic fibrosis the ΔF508 mutation has a frequency of 74.5%; evaluating for this and the 15 other most "common" mutations gives a detection rate among North American whites of approximately 86%.[1] Thus, direct detection still will not detect 100% of individuals with a cystic fibrosis allele. In Gaucher's disease, four mutations account for nearly 96% of the Gaucher's disease mutations in the Jewish population.[2]

In cases where there is a family history of a particular disease and direct analysis of mutations fails to detect any documented mutation, indirect analysis by linkage with polymorphic markers is usually a reliable alternative. Analysis of polymorphisms is successful if the polymorphism employed is closely associated with the disease allele within a family or if it is specifically not associated with a disease allele. Although

such indirect detection techniques are useful, they also increase difficulty of analysis owing to (1) the need for testing several (often many) family members, (2) nonpaternity, or (3) parental polymorphisms uninformative at a particular locus.

One strategy for nearly 100% detection of any particular disorder is the use of indirect detection when direct detection fails. In our cystic fibrosis example, after analysis of common mutations, linkage analysis of uncommon ("private") mutations—currently greater than 400—may be used if enough family members are available for study. Unfortunately, given the current limits on available technology, assay for all documented mutations that cause cystic fibrosis is impractical, due to the cost and labor involved. This is the basis for recommendations against general-population screening (ie, in persons without affected first-degree family members).

Techniques Used in DNA Analysis

Some techniques commonly used in DNA analysis are described briefly below. For detailed information, consult the appropriate references cited. An excellent source for all molecular genetics–related methods is the three-volume laboratory text by Sambrook, Fritsch, and Maniatis.[3]

DNA isolation

One reason for the rapid application of molecular genetic techniques in DNA diagnostics has been the ease of obtaining DNA from virtually any tissue or fluid, owing to the stable nature of DNA and the small quantity required for most DNA diagnosis. DNA is present in all nucleated cells, and each cell contains DNA comprising the whole of an individual's genetic constitution. DNA can be extracted from blood (leukocytes), amniotic fluid (amniocytes), chorionic villi samples (CVS), and all other tissue types.

The extraction procedure involves the collection of cells from the fluid by centrifugation, followed by disruption of the cellular wall, then either digestion of all proteins by proteinase K or selective precipitation of proteins by salts. The DNA remains soluble, and the DNA-containing solution is mixed with organic solvent in the presence of salt to precipitate the DNA. The DNA is further purified in a few steps to yield pure DNA suitable for all types of manipulation.

Purified DNA, in the absence of DNases (enzymes that selectively break down DNA), is stable and can be stored in solution form at –20°C or –70°C. Repeated freeze-thaw cycles tend to break down high-molecular genomic DNA into smaller fragments of 50 to 100 kb in size.

Table 1 shows the approximate amount of DNA present in various tissue samples. A comparison is also given in terms of number of copies of the human genome to expect when sample content is termed in weight units.

Hybridization analysis

Hybridization is the basis of nearly all DNA diagnostic methods. The seminal discovery by Watson and Crick in 1953[4,5] established the double-stranded, helical structure of DNA as well as the A-T and G-C base pairings on which the principles of hybridization studies are founded. The specificity of complementary base pairing is such that a small oligonucleotide sequence (*probe*) will identify and *hybridize* to its complementary sequence. This hybridization process occurs in less than 30 seconds under appropriate conditions and is the principle behind the polymerase chain reaction (PCR), described in the next section.

Hybridization detection methods can be divided arbitrarily into two groups: gross detection and nucleotide level detection.

In *gross detection*, hybridization involves DNA probes greater than 200 bp in length (even though oligonucleotides can be used). The resolution achieved may relate to the detection of DNA fragments on a gel (bands) representing a particular size, such as in the restriction fragment length polymorphism (RFLP) used in assays for fragile X, or the absence of certain bands, representing deletions, as is employed in Duchenne's muscular dystrophy (DMD) testing.

Gross detection is achieved using Southern blot analysis, a technique developed by E.M. Southern.[6] It is a method based on hybridization of DNA probes in solution to complementary sequences immobilized on membrane after electrophoretic separation. Variations of this technique are Northern blot analysis, where RNA is bound to membranes after electrophoretic separation; Western blot analysis, an immunologic-based

Table 1
DNA Content

Sample	Amount
One cell	~7 pg
One plucked hair	~0.3 μg (300 ng)
One shed hair	~0.1 ng
One drop blood	~1.5 μg
One drop semen	~10 μg
1 mL blood	~40 μg
1 mL amniotic fluid	~0.35 μg
1 mg chorionic villi	~1 μg
One T25 flask cultured cells	~30 μg
Human genome	3 billion bp (haploid)
1 μg genomic DNA	333,000 copies
10 ng	3300 copies
6 pg	Single copy of genome

assay; and dot blot analysis, where DNA or RNA is bound to membranes by direct application of the sample material without electrophoretic separation.

These blot analysis methods are powerful tools for identifying gene sequences. In the context of DNA diagnostics, the aim is to locate a specific DNA fragment corresponding to a particular gene linked to a particular phenotype based on RFLP hybridization, using probes that are specific for the particular gene fragment or markers that detect the RFLP employed. The size of the probes used for Southern analysis is usually 200 bp or greater; however, probe size does not bear any direct effect on the hybridization. In Southern hybridization, conditions are such that the technology cannot discriminate between single base-pair mismatches or small deletions—a limitation of the technique.

To detect a particular fragment of human DNA, the genomic DNA is cut into small pieces with restriction endonucleases, bacterial enzymes that cleave DNA at specific sites based on its sequence specificity. The presence of these specific 6 to 8 bp sequences in the human genome is random; thus, digestion of genomic DNA with any restriction enzyme leads to the generation of millions of fragments of all sizes. No particular size is more enriched than another. To separate the different-sized fragments, the digested DNA is subjected to electrophoresis.

Visualization of genomic DNA after digestion with restriction endonucleases and electrophoretic separation reveals a lane with a smear for each DNA sample (Fig 1A). Transfer of the separated DNA to membranes capable of binding DNA is achieved either by capillary blotting techniques or by vacuum-assisted methods that accelerate transfers. The transferred and immobilized DNA, now affixed on the membrane, is a mirror image of that noted in the gel. Membrane hybridization with specifically labeled probes leads to the specific annealing/binding of the probe only to its complementary band. The hybridization is detected by autoradiography, in the case of radioactive probes, or by chemiluminescent methods (Fig 1B).

For *nucleotide level detection*, hybridization conditions that can discriminate between single base-pair mismatches are used. This is usually performed with dot blot analysis, in which the probe size is 18 to 30 bp. These probes are called allele-specific oligonucleotides (ASOs), also commonly called oligos. ASO hybridization conditions are achieved by either adjusting the temperature of hybridization or by adjusting the salt concentration. ASO hybridization is rapidly gaining use in the detection of all single base-pair mutations as well as small deletions. The advantage of dot blot analysis is the large number of samples that may be processed simultaneously. The drawbacks of ASO hybridization are the use of radiolabeled probes and the requirement of thorough optimization of hybridization conditions to discriminate between single base-pair mismatches. As nonradioactive detection methods become more available, ASO techniques will rapidly become the principal method of detection for most mutations. The use of ASO will be discussed in the section on cystic fibrosis.

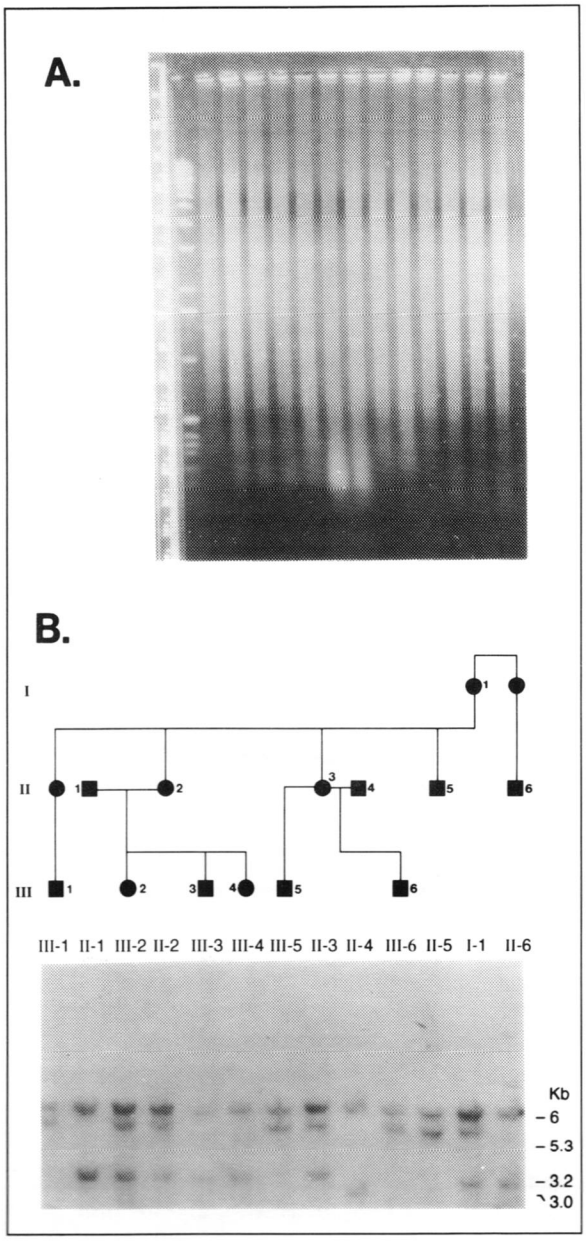

Fig 1.

Southern blotting and restriction fragment length polymorphism.

A. Genomic DNA digested with a restriction endonuclease, followed by electrophoretic separation. The first lane is molecular-weight-marker control lane; all other lanes show a smear signifying genomic DNA cleaved at numerous sites with no particular fragment more intense than another.

B. The same gel was blotted onto a membrane and processed for hybridization with a unique, specific probe. The probe hybridizes only to fragments complementary to it. The pattern clearly establishes a particular genotype in a pedigree.

Polymerase chain reaction

The polymerase chain reaction (PCR) is the single most commonly used procedure in molecular genetics. PCR was developed in 1985[7] and, due to its ability to amplify specific regions of DNA several million-fold, has since become the major contributing factor in the rapid pace of research in molecular genetics.[8,9]

PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two complementary, short, oligonucleotide primers whose sequence is known. These primers are designed such that each corresponds to one of the strands; the distance between these primers limits the amplification fragment size. PCR is also based on the property that in hybridization the primer, under specific conditions, will bind (hybridize, anneal) *only* to its cognate sequence in the DNA sample. This process is termed *annealing*. The annealing of the primer to target DNA is achieved by denaturing the target DNA through exposure to high temperature. Heat breaks the base-pair hydrogen bonds and results in a separation of the two DNA strands. Reducing the temperature favors base pairing/hybridization, which is equally competed for by the primers, present in excess.

Once the primers are correctly annealed, DNA polymerase will elongate the primer by copying the template sequence. The two primers are both extended beyond the binding site, independent of each other, resulting in the synthesis of variable-size fragments complementary to the template DNA. The steps of denaturation, annealing, and elongation constitute one cycle. In one cycle, a copy of the target sequence is achieved. The second cycle of amplification yields four copies, and so forth. The amplification process occurs exponentially and can result in the amplification of a target sequence several million-fold. A routine PCR process consists of 20 to 30 cycles, requiring 2 to 3 hours and using an automated thermal cycling instrument. Under proper conditions, a unique gene sequence from the genome can be routinely amplified from 50 to 100 ng of target DNA. PCR has been used to amplify sequences from one cell (approximately 7 pg DNA) and a single hair (300 ng).

The use of *Taq* DNA polymerase was crucial to the rapid and exponential popularity of the technique.[10,11] Initially, when PCR was first introduced, a thermolabile DNA polymerase was used; unfortunately, the warm denaturation temperatures (92°C to 94°C) inactivated the enzyme. Those active in the field may remember sitting in front of three water baths, adding aliquots of DNA polymerase after each annealing cycle, and transferring the tubes to another water bath. The heat-stable *Taq* DNA polymerase (an enzyme acquired from hot-spring bacteria) can withstand repetitive exposure to the warm temperatures employed in the thermal cycling process with little loss of activity, thereby eliminating the need to add more enzyme after each cycle. The use of *Taq* DNA polymerase also facilitated the introduction of automated DNA thermal cyclers.

The rapid amplification of sequences of specific portions of the gene spanning the site of the mutation, coupled with other detection methods, has led to an expanded availability of DNA diagnostic methods in clinical medicine. For example, PCR can also be used for mRNA amplification by first making a complementary DNA copy that serves as the target DNA. DNA complementary to mRNA is synthesized by the use of an enzyme called reverse transcriptase (RT); the process of using mRNA → cDNA → PCR is called RT-PCR. RT-PCR is valuable when the exact mutation is not known and techniques for scanning mutations in short fragments is required, eg, using single-strand conformation polymorphisms.[3]

Polymorphism Analysis

We earlier discussed the heterogeneity of the human genome. In this section we will examine how the application of these DNA polymorphisms may be exploited for linkage analysis in the detection of a genetic disorder.

Two types of polymorphisms will be discussed: restriction fragment length polymorphisms (RFLPs), which have already been introduced, and microsatellite repeat polymorphisms. Both these techniques have contributed immensely to the human gene mapping effort. Although RFLP analysis has been in use for well over a decade, microsatellite repeat mapping is considerably more recent.

Restriction fragment length polymorphism analysis

RFLP analysis is based on the observation that changes in the DNA create or abolish cleavage sites for restriction endonucleases. Botstein et al[12] first identified the existence of RFLPs and advocated their use as markers for linkage analysis. Since then, extensive screening of the human genome has been accomplished by digestion with various enzymes, followed by "probing" with specific cloned pieces of DNA. This methodology has established a data base, DNA markers, of polymorphic sites of differing fragment sizes obtained following specific enzymatic cleavage.[13,14] As noted earlier, a specific chromosomal locus is termed polymorphic when there exist two or more alleles and each has a population frequency of greater than 1%. For RFLP analysis, polymorphic alleles are selected that will provide a heterozygote frequency of at least 20%. RFLPs are essential for the deduction of allelic inheritance, but are useful only when the parents are informative. The deduction of inheritance of a particular phenotype using a specifically linked polymorphism is complex, most com-

monly established by thorough and laborious effort in research-oriented laboratories. As noted earlier, RFLPs are most useful when methods of direct detection of common mutations have failed (presumably due to the presence of an undocumented private mutation). Indirect analysis made by deduction of allelic inheritance is demonstrated in Fig 1B.

Microsatellite repeat polymorphisms

Microsatellite repeat polymorphism analysis is rapidly replacing the use of RFLPs for gene mapping. Microsatellite repeat polymorphisms are small repeats, eg, CA (dinucleotide repeat), that are interspersed approximately every 30 to 50 kb within the human genome. Weber and May in 1989[15] demonstrated the existence of polymorphic microsatellites with alleles ranging from 4 to 11 bp. The abundance and the highly polymorphic nature of microsatellite repeat polymorphisms make it ideal for use as a mapping technique for allele inheritance patterns. Microsatellite repeat mapping technique involves PCR, usually in the presence of a radioactive label, followed by electrophoretic separation on a sequencing gel. Microsatellite repeat mapping is more versatile, powerful, and less time-consuming than RFLP.

Clinical Applications of Molecular Genetics

The following sections discuss the molecular genetics of several common inherited diseases. Also described are the methods of molecular analysis used to determine the presence of genetic mutations, which are in turn used to assess an individual's risk of developing the disease. The case presentation format illustrates the practical applications of molecular genetic technology.

Sickle Cell Disease

CASE. A couple presents for genetic counseling because the mother, who is 10 weeks' pregnant, has a child from a previous marriage affected with sickle cell disease. The father, a colonel in the army, is unavailable for testing due to his deployment with NATO forces in Bosnia. Although the child has experienced few crises, all have necessitated hospitalization and repeated blood transfusions. Recently, he demonstrated seropositivity to the human immunodeficiency virus (HIV).

Molecular genetics

The study of sickle cell anemia and other β-globin disorders holds special importance in the fields of medicine, physiology, biochemistry, and molecular genetics. During the early 1940s, several groups independently started working on the genetics and biochemistry of sickle cell anemia. In 1949, Neel[16] showed that sickle cell anemia fits the pattern of a genetic disease and is caused by the presence of two copies of a recessive allele, thus causing the disease in the homozygous state. Later the same year, Pauling et al[17] showed that hemoglobin in normal and sickle cell anemia patients differed by having different electrophoretic mobility and, thus, different chemical properties. In 1957, Ingram[18] analyzed the α- and β-globin chains of adult hemoglobin obtained from both normal subjects and patients with sickle cell anemia. In the latter group, he was unable to demonstrate changes in the hemoglobin α chains; however, he found that each β chain had an amino acid substitution at position 6, which resulted in a mutation from the normal ("wild-type") glutamic acid residue to valine. This was the first report of a mutation being identified at the protein level as a cause of an inherited disease; Ingram's work also made apparent the fact that subtle changes at the molecular level may lead to a clinically distinct disorder.

β-Globin was the first gene to be cloned and completely sequenced. The glutamic-acid-to-valine substitution has now been further characterized, and the mutation at the DNA level has been established to be an A to T change in the second position of the codon of glutamic acid, ie, GAG to GTG. The resulting mutant globin chain is termed hemoglobin S (HbS). Hemoglobin S is freely soluble when fully oxygenated. Under conditions of low oxygen tension, the red cells become grossly abnormal, assuming a sickle shape that leads to aggregation and hemolysis. Homozygous HbS is a serious hemoglobinopathy found almost exclusively in the black population. About 8% of African Americans are carriers—heterozygotes—and about 0.2% are affected—homozygotes. Heterozygotes (sickle cell trait) are clinically normal, although their red cells will sickle when subjected to very low oxygen pressure in vitro.[19]

DNA testing for the sickle cell mutation is accomplished by specific amplification of the region spanning the mutation, using polymerase chain reaction, followed by enzymatic cleavage of the amplified product. Sickle cell mutation eliminates a restriction endonuclease site (*Dde* I), and electrophoretic resolution of the fragment pattern reveals the presence or absence of the mutation. Clear diagnosis of normal, carrier, and homozygous DNA is readily achieved.

Molecular analysis

The example of sickle cell mutation detection illustrates how creation or abolition of a restriction endonuclease site may be due to a mutation. All mutations, however, are not as easily determined; for example, some mutations may not be in a DNA sequence region recognized by any known restriction endonuclease. In such cases, an artificial restriction

site may be introduced by mismatch primer construction, which can then discriminate between wild-type normal and mutant sequence. This is discussed with examples under Gaucher's disease.

In sickle cell disease, the A to T base mutation abolishes a cleavage site for restriction endonuclease *Dde* I and *Mst* II.[7] To detect the sickle cell mutation using the *Dde* I restriction enzyme, two specific oligonucleotide primers are used to amplify a 110-bp fragment within the β-globin gene by PCR. The A to T mutation and the *Dde* I site are in the middle of the fragment. After PCR amplification, the amplified product is subjected to restriction endonuclease *Dde* I digestion; only the β^A-globin fragment is cut by *Dde* I to yield two 55-bp fragments. Heterozygous individuals with both a β^A- and a β^S-allele will yield fragment patterns in which half the molecules cleave to 55 bp and half fail to cleave, leaving a 110-bp fragment. Homozygous affected individuals will have only β^S-globin, and thus the PCR-amplified, 110-bp fragments from both alleles will have lost the *Dde* I site and will remain uncleaved. The PCR-amplified, *Dde* I-digested DNA is electrophoresed to resolve the fragments. Visual inspection of the gel (see gel picture in Fig 2) for DNA fragment pattern reveals the presence or absence of the mutation in question. Clear diagnosis of normal, carrier, and homozygous DNA is achieved. Fig 2 shows the fragment pattern obtained for sickle cell using the above detection method.

Alternate methods using allele-specific oligonucleotide and Southern blotting techniques with *Mst* II- or *Dde* I-digested DNA and hybridization with a β-globin gene probe have also been reported.[7,20,21]

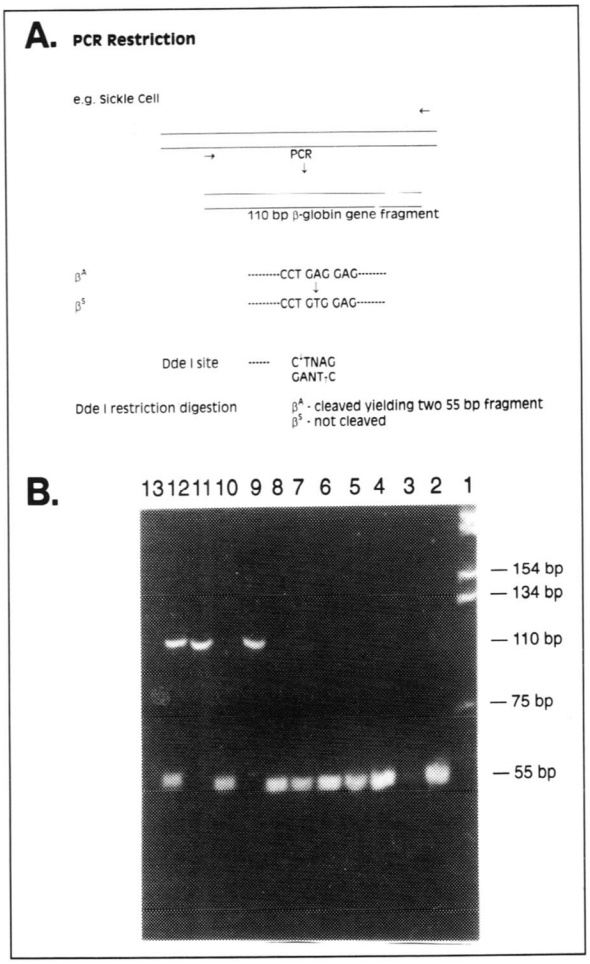

Fig 2.

Top portion of figure depicts the amplification of β-globin-specific, 110-bp fragment spanning the sickle cell mutation site. Cleavage of the PCR-amplified fragment with restriction endonuclease *Dde* I will result in the generation of two fragments for products of β^A-globin gene. β^S-globin specific gene product is not cleaved by *Dde* I, as the A to T base change has abolished the site. Heterozygous individuals will have the β^A-allele product cleaved and the β^S-allele product resistant to cleavage. The gel photograph is of β-globin-specific PCR product digested with *Dde* I.

Lane 1, molecular-weight marker; lanes 2 to 8 and 10, β^A-homozygous individuals; lanes 9 and 11, β^S-homozygous individuals; lane 12, β^A-and β^S-heterozygous individuals.

CYSTIC FIBROSIS

CASE. A couple presents to your office for genetic counseling because the mother and their two only children have cystic fibrosis. They are hopeful about the possibility of having a child unaffected with this condition.

Molecular genetics

Cystic fibrosis is the most common recessive disorder affecting the white population, with a heterozygote frequency of approximately 1 in 25.[19] Recently, the gene responsible for cystic fibrosis has been identified: the cystic fibrosis transmembrane conductance regulator (*CFTR*). Rapid characterization of the mutations and their frequency has made possible DNA testing for *CF* in at-risk families.[1]

Table 2
Frequency of *CF* Carrier

White Americans of European descent	1 in 25
Ashkenazic Jews	1 in 29
Hispanic Americans	1 in 45
African Americans	1 in 60
Asian Americans	1 in 150

A single mutation, termed ΔF508, accounts for nearly 75% of *CF* mutations in the whites of northern European descent (Table 2). This particular mutation is due to an in-frame deletion of three bases, resulting in the deletion of amino acid phenylalanine (F) at position 508.[1] Most of the other mutations are single base changes resulting in amino acid substitution. Table 3 lists some of the mutations. The *CFTR* gene has been thoroughly characterized, and the position of most mutations in the gene in relation to protein structure/function have been established; details are beyond the scope of this discussion.

Table 3
***CF* Mutation Analysis**

White (non-Jewish)	Ashkenazic Jews
ΔF508 (75.8%, exon 10)	W1282X (60%, exon 20)
G542X (2.7%, exon 11)	ΔF508 (23%, exon 10)
G551D (3.2%, exon 11)	G542X (4%, exon 11)
R553X (1.4%, exon 11)	N1303K (4%, exon 21)
N1303K (1.4%, exon 21)	3849+10 kb C-T (4%)

Analysis of five of the most common mutations (see Table 3) accounts for about 85% in the white population.[1] The frequency of ΔF508 mutation in Ashkenazic Jews is approximately 23%, whereas W1282X mutation in exon 20 represents 60% of the mutations; W1282X mutation leads to a truncated protein. The five mutations listed in the table for the Ashkenazic population account for nearly 96% to 98%.[22]

Molecular analysis

Development of new mutation analysis methods for cystic fibrosis has been very active, primarily because of the high carrier rate and also due to carrier screening prospects. The traditional sample for DNA is blood or amniotic fluid/culture for prenatal diagnosis. Recently, methods have been reported for DNA prepared from buccal brushing/swab, followed by multiplex PCR amplification and ASO hybridization.[23,24] In this section we will discuss PCR/restriction and ASO methods.

Nearly all the mutations analyzed in the evaluation of mutant *CF* alleles either create or abolish a restriction endonuclease site, with the exception of the ΔF508 mutation—a 3-bp deletion. To analyze the mutations, specific corresponding fragments of the *CFTR* gene are amplified by PCR. For the ΔF508 mutation, the product after PCR will be 3 bp shorter than the normal (wild-type) sequence. Electrophoresis of PCR-amplified fragments from homozygous normal, heterozygous, and homozygous ΔF508 will reveal fragment patterns in which the homozygous normal and the homozygous affected will manifest only one specific band of the amplified product.

In heterozygotes (carrier) individuals there will be two fragments, one of which is 3 bp smaller than the other. The smaller fragment represents the ΔF508-deleted allele; the larger band represents the normal allele.

In affected individuals—ΔF508 homozygotes—there will be a single band with the same mobility as the smaller band in the heterozygous sample. This single band actually represents two fragments of the same size traveling together, amplified from the two ΔF508-deleted allele segments. Analysis of the ΔF508 mutation is straightforward, involving PCR specific for exon 10 ΔF508 mutation-spanning region, followed by polyacrylamide electrophoresis. The interpretation of the gel patterns is clear and reliable.

For other mutations of the *CFTR* gene, the strategy is similar to that described above for sickle cell disease. Following specific PCR amplification, the PCR product is subjected to restriction endonuclease digestion to monitor cleavage of the DNA. Table 4 lists the different enzymes used and the expected fragment-size products on electrophoresis.

Allele-specific oligonucleotide analysis

Another powerful technique used for mutation detection is allele-specific oligonucleotide (ASO) hybridization.[25] In this technique, small oligonucleotides in the size range of 18 to 26 bp are synthesized corresponding to the normal and mutant DNA sequence. The normal and mutant ASOs differ by only 1 bp. These oligonucleotides are usually end-labeled with ^{32}P and used for very specific hybridization and washing conditions. Under appropriate conditions, the normal ASO will hybridize only to the wild-type sequence, and mutant ASO, only to the mutant sequence. The labeled ASOs are used as probes to hybridize with PCR-amplified DNA fragments corresponding to each mutation-spanning region. The DNA fragments are applied to a DNA-binding membrane in duplicate as dots or slots, using

Table 4
Cystic Fibrosis PCR/Restriction-Based DNA Analysis: Fragment Sizes

Mutation	Restriction enzyme	PCR product (bp)	Normal (bp)	Homozygous (bp)	Carrier (bp)
ΔF508			79/79	76/76	79/76
G542X	*Bst* NI	114	90+24	114	114+90+24
G551D/R553X	*Hind* II	114	55+59	114	114+55+59
N1303K	*Bst* NI	60	40+20	60	60+40+20
W1282X	*Mnl* I	473	178+172+123	301+172	301+178+172+123
CT3849	*Hph* I	437	349+88	222+127+88	349+222+127+88

a special apparatus called a *dot/slot blot apparatus*. This apparatus has exact-size holes to make all applications uniform in size. The duplicate membranes are each hybridized to normal and mutant ASO; the result after hybridization is tabulated by scoring for absence or presence of hybridization with each ASO. Hybridization with only the normal ASO signifies the presence of normal sequence for that mutation; positive signal hybridization with both normal and mutant ASO indicates a heterozygous sample; and positive hybridization only with the mutant ASO signifies homozygous abnormal status. The use of ASO hybridization requires *exact* optimization of the melting temperature and washing conditions to discriminate between single base-pair hybridization. If properly performed, the ASO hybridization technique holds the most promise in terms of speed, ability to handle a large number of samples, and per-sample cost.

GAUCHER'S DISEASE

CASE. A teenager and her mother present to your clinic for counseling and therapy for osteonecrosis of the femoral head, noted on an admission radiograph during her recent hospitalization for anemia, thrombocytopenia, and hypersplenism. Her clinical symptoms predominantly include bone pain, which requires bed rest and absence from school. She has had two episodes of pneumonia, also requiring hospitalization.

Molecular genetics

Gaucher's disease is the most common glycolipid storage disease; it is due to a deficiency of glucocerebrosidase. Gaucher's disease has, in the Jewish population, an estimated heterozygote frequency of approximately 9%.[2] The gene has been cloned and characterized. A pseudogene is also present, which complicates DNA analysis (discussed later).

The four mutations listed in Table 5 account for approximately 96% of the Gaucher's disease mutations in the Jewish population.[26] Mutation 1226G is the most common cause of Gaucher's disease in Jewish patients and is associated with mild, late-onset clinical phenotype. Only about one third of patients with the 1226G/1226G mutations have Gaucher's disease. Patients who are compound heterozygotes for mutations 1226G and 84GG have a more severe clinical disorder than those who are homozygous for the 1226G mutation. The median age at first onset of symptoms in patients with Gaucher's disease having the 1226G/1226G or the 1226G/84GG mutation is 30.5 years and 6 years, respectively. There has been no report of patients homozygous for the 84GG mutation, indicating that it would be a perinatal lethal condition. Mutation 1448C is associated with a more severe phenotype compared with the 1226G mutation. Patients with 1448C/1448C genotype generally manifest severe neuronopathic Gaucher's disease; patients with a homozygous IVS2 mutation are also severely affected.

Table 5
Gaucher's Disease Mutation Analysis

Mutation	Frequency	
1226G (N370S)	75%	(25% non-Jewish)
84GG	13%	
1448C (L444P)	5%	(40% non-Jewish)
IVS2+1	3%	

Beutler and colleagues[26] identified a total mutation frequency of approximately 0.031 in the Ashkenazi Jewish population. The frequency of the 1226G mutation is about 0.028, and that of the 84GG mutation is 0.0028. Thus, the frequency of alleles other than 1226G, 84GG, and 1448C would be 3.3% of the total, or 1×10^{-3}. A Jewish couple who is negative for the 1226G, 84GG, and 1448C will therefore have only an approximately 1:1,000,000 risk of having a child with Gaucher's disease. On the other hand, if one partner has one of the three "common" mutations and the other none of these three, then the risk will be increased to approximately 1:1000.

Molecular analysis

DNA analysis for Gaucher's disease mutations is accomplished by specific PCR amplification, mismatched PCR (discussed below), followed by restriction endonuclease digestion and/or ASO hybridization. We previously discussed PCR followed by restriction endonuclease digestion and ASO. Here we will discuss a new method of mutation detection, *mismatched PCR.*

Not all mutations result in the gain or loss of a restriction site. Such mutations therefore cannot be analyzed by PCR/restriction endonuclease method. ASO requires the use of radioactivity and thorough optimization. The mismatched PCR method was introduced by Beutler et al[27] to overcome these difficulties. In this method, one of the primers for PCR is constructed in a way that the 3′ end of the DNA strand—adjacent to the site of the mutation and the internal sequence of the primer—is altered so that a restriction endonuclease site will either be gained or lost once the PCR product is amplified.

The example given in Fig 3A is for Gaucher's disease mutation 1226G (also known as N370S). In this mutation, an A is changed to a G at position 1226, leading to the substitution of the amino acid serine for asparagine. This mutation does not create or abolish a site for any known/commercially available restriction endonuclease.

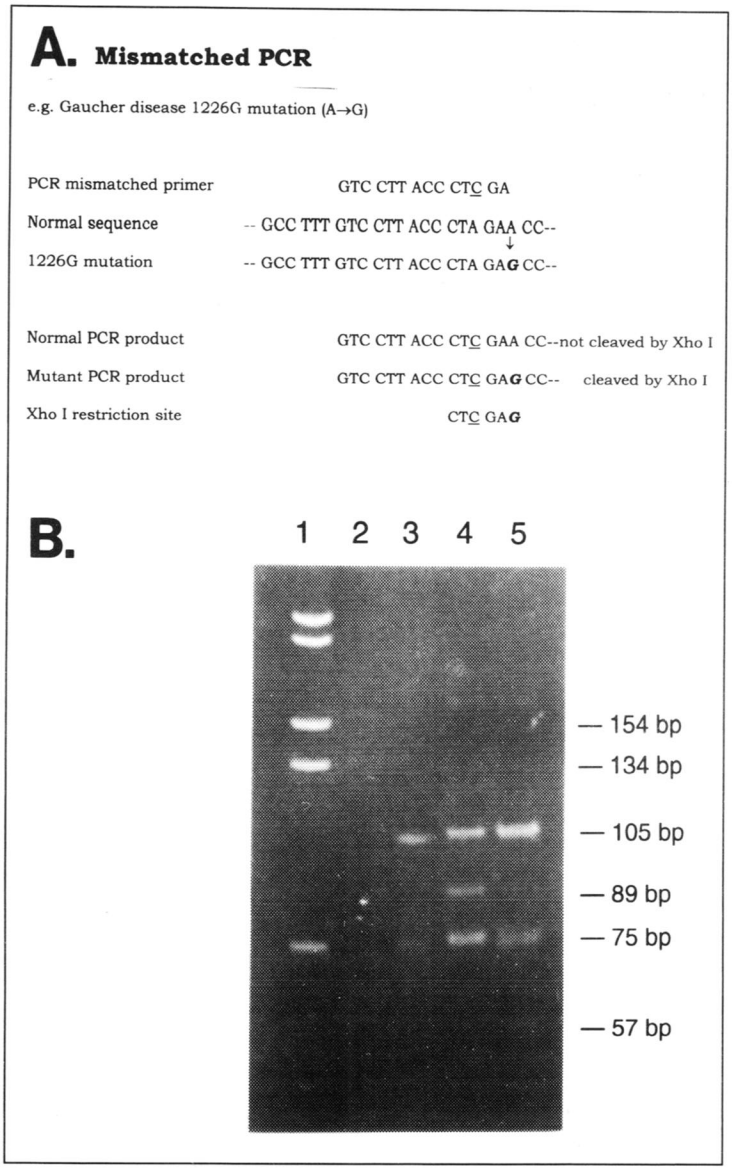

Fig 3.
Mismatched PCR is based on the planned mismatched construction of one of the primers such that, after elongation during the PCR reaction, the product gains a restriction site either in the normal or mutant product. In this example, an *Xho* I site is created in the mutant product. Digestion of the PCR product, followed by electrophoresis and visualization, will indicate the status of the particular sample.

One of the PCR primers is constructed with a mismatch, as shown in Fig 3B. Primers with internal mismatches will hybridize to target sequences at optimized conditions, and elongation of this primer with a normal template will result in the addition of an A residue; in the mutant template, a G residue will be added. The use of the mismatched primer in concert with a 1226G mutant template creates a new *Xho* I restriction endonuclease site (Table 6). Digestion of PCR products from normal and 1226G mutant templates is followed by electrophoretic separation. The result will be two fragments for the mutant product (it will be cleaved), whereas the normal product remains uncleaved, resulting in visualization of a single, higher-molecular-weight fragment. This technique is reliable, and it is performed in a fashion very similar to PCR, followed by restriction endonuclease digestion. This mismatched PCR method may also be used for the 84GG Gaucher mutation.[27]

Fragile X Syndrome

CASE. A couple presents for genetic counseling because of a family history of mental retardation. The affected girls are described as "slow." Some of the boys in the family are "slower than the girls," some were hyperactive (autistic) as children, and some of the "slow" men are reported to have large, flat ears.

Molecular genetics

Identification of the *FMR1* gene and establishment of its expansion as the cause of fragile X syndrome led to the classification of a new class of mutation[28,29]: Fragile X syndrome is associated

Table 6
Gaucher's Disease PCR/Restriction-Based DNA Analysis: Fragment Sizes

Mutation	Restriction enzyme	PCR product (bp)	Normal (bp)	Homozygous (bp)	Carrier (bp)
1226G	*Xho* I	105	105	89+16	105+89+16
84GG	*Bsa* BI	75	75	57+18	75+57+18
1448C (1st)	*Pst* I	677			
1448C (2nd)	*Nci* I	102	102	57+45	102+57+45
IVS2+1	*Hph* I	357	141+117+99	240+117	240+141+117+99

with amplification of a triple-repeat CGG in the *FMR1* gene, the severity of the disease being related to the size of the amplification. The genetics of fragile X syndrome are complicated, but may be better understood if one groups the expanding mutations into two broad categories: *premutations* and *full mutations*.

Premutations are found in normal transmitting males (NTMs), individuals who transmit the mutation to grandsons but are unaffected themselves, and carrier females. Premutations involve the amplification of the CGG triple repeat to approximately 70 to 200 copies. Numbers of repeats in this range are considered stable. Normal individuals possess less than approximately 50 copies of the triple repeat, and individuals with full mutations of the fragile X syndrome have 200 to 1000 copies of the CGG triple repeat. The size of expansion is heterogeneous within an individual and thus signifies somatic *instability* of the mutant allele.

The mode of transmission of the fragile X mutation is unusual and makes both understanding the genetics and counseling patients difficult. Phenotypically normal males possessing a premutation are normal transmitting males, as noted above, and father-to-daughter transmission is *not* accompanied by expansion of the triple-repeat mutation. Thus, daughters of NTMs are never found to be affected.

The change from premutation to full mutation occurs only in females and may be, through them, transmitted to their offspring. The risk of expansion of a premutation to a full mutation varies, depending on the size of the premutation. This new class of triple-repeat expansion mutation has now been documented in several other genetic disorders, including Huntington's disease and myotonic dystrophy.[30]

Molecular analysis

The detection of DNA amplification/expansion regions may be accomplished by PCR and Southern hybridization. The following methods can be used for all disorders involving a variable increase in the size of a specific region of DNA. Analysis for the direct detection of fragile X mutation is based on the enzymatic amplification of a fragment containing the CGG repeat sequence of the *FMR1* gene, and it is most commonly performed with a modification of the amplification protocol published by Fu et al.[31]

This protocol detects the fragile X mutation by the size of the amplified product. An increase in size is correlated with the corresponding number of CGG repeats, following which a risk is calculated. The most common allele in the unaffected, normal population consists of 29 repeats, the range varying from 6 to 54 repeats. Premutations in fragile X families showing no phenotypic effect range in size from 52 to greater than 200 repeats; however, all alleles with greater than 52 repeats are meiotically unstable.

PCR-based methods are fundamentally similar to those presented earlier in this article. The two primers are constructed such that they span the region of triple-repeat expansion; however, in the case of fragile X specifically, the nature of the mutation poses problems using normal PCR conditions: the CGG repeat may be hundreds to thousands of bases in length. All DNA polymerases, including *Taq* DNA polymerase, do not efficiently copy prolonged stretches of G residues. Therefore, in fragile X studies, an analog of G (7-deaza GTP) functions more efficiently and is therefore incorporated into the PCR reaction to achieve optimal amplification. Unfortunately, the use of 7-deaza GTP mixtures precludes the staining of gels with ethidium bromide, minimizing visualization (7-deaza GTP containing DNA does not stain well). The poor staining is resolved by using radioactively labeled nucleotide, followed by autoradiography.

Fragile X PCR still does not give accurate results for full mutations due to the presence of massive CGG triplet expansions, because, as noted above, PCR does not amplify very large fragments containing repetitious G residues efficiently. Although normal and premutation PCR amplifications are reliable, all amplifications performed in our laboratory on subjects who may possess full mutations are run by *both* PCR and Southern hybridization techniques. The PCR results are obtained in 2 days; Southern blot hybridization studies require more time for complete results.

Southern blot analysis for fragile X mutation detection involves the cleavage of DNA with enzymes *Eco* RI and *Eag* I and is based on the protocol published by Rousseau et al.[32] The Rousseau method identifies the size of CGG repeat region and accomplishes this by hybridizing probe StB12.3 to DNA that has been previously double-digested with restriction enzymes *Eco* RI and *Eag* I. The sample is then blotted onto a membrane.

In normal females two fragments are seen, a 2.8-kb fragment corresponding to the active X and a 5.2-kb fragment corresponding to the methylated, inactive X chromosome. Normal males exhibit only the 2.8-kb banding pattern. Affected males will have an amplified CGG repeat region with methylation, thus giving rise to fragments larger than the normal 5.2 kb. Premutations in both males and females will be seen as 2.9- to 3.3-kb fragments (normal, 2.8 kb) derived from the active X chromosome. Premutations in females derived from the inactive X will manifest fragments from 5.3 to 5.7 kb in length. Mosaicism is characterized by fragments appearing as a mixture of full mutation (methylated, larger than 5.7 kb) and unmethylated premutation (2.9 to 3.3 kb) fragments (Fig 4).

DUCHENNE'S MUSCULAR DYSTROPHY

CASE. The final case in your morning clinic is a 13-year-old boy who has been experiencing progressive muscular weakness and pseudohypertrophy of the calf muscles. His mother is concerned about recurrence risks for this condition in her future pregnancies.

Molecular genetics

The dystrophin gene is the largest gene thus far identified, being approximately 2300 kb (2.3 million base pairs) in size. Almost 50% of all patients with Duchenne's muscular dystrophy (DMD) have deletions in the dystrophin gene, likely due to its unusually large size. In addition, the dystrophin gene has an exceedingly great number of new mutations, also attributed to size. Finally, one third of these mutations are new, while those remaining are inherited through heterozygous females; DMD is lethal in males.

Becker type muscular dystrophy (BMD) is an allelic form of DMD and is due either to mutations in the dystrophin gene, which do not cause total loss of protein function, or to deletions that do not cause change of reading frame. *Frame-shift mutations* are small deletions that cause a shift in the reading frame, leading to production of a truncated gene product. *In-frame deletions*, on the other hand, result in the removal of a portion of the amino acid sequence, thereby allowing some retention of functional activity.[19]

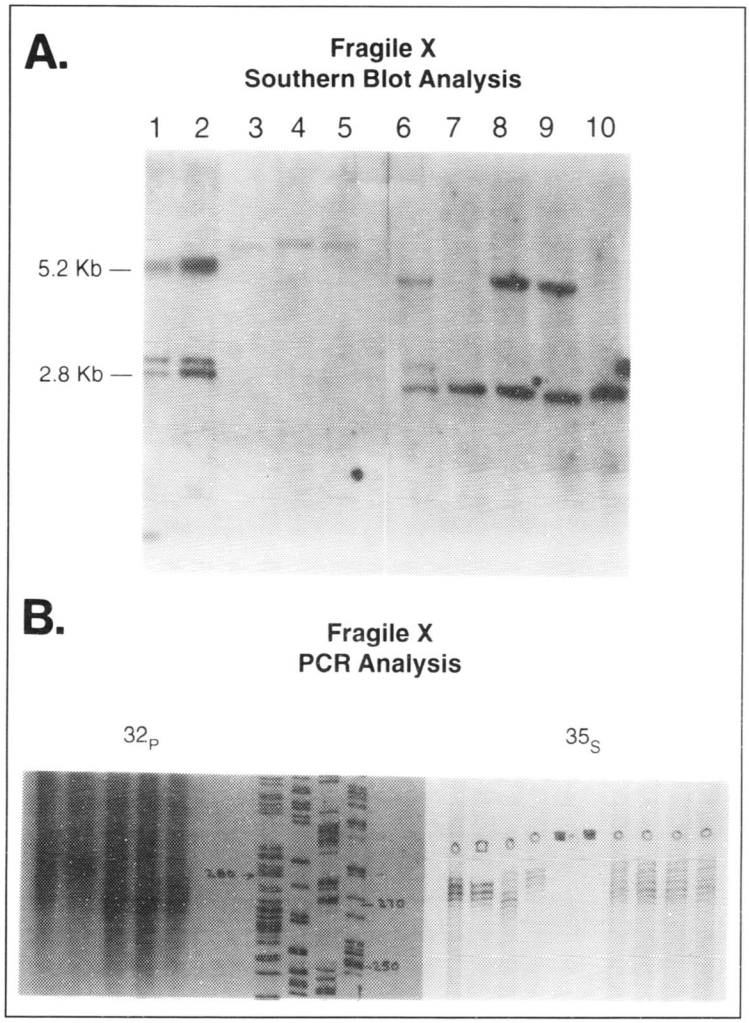

Fig 4.

Fragile X detection methods.

A. Southern blot analysis. Genomic DNA digested with *Eco* RI and *Eag* I, Southern blotted and hybridized with fragile X probe FXAJS. A distinctive pattern is observed (see text).

Lanes 1, 2, and 6 represent females with a premutation; lanes 8 and 9, normal female pattern; lanes 3, 4, and 5, males with full mutation; lanes 7 and 10, normal male pattern.

B. PCR analysis. The triple-repeat pattern with size marker is shown. Full mutations cannot be detected normally by PCR analysis.

Molecular analysis

The dystrophin gene has more than 70 exons. As a result, the usual approach to detecting base-pair-level mutation is not practical. Therefore, with DMD, the initial goal is screening for deletions.

Studies of regions where there is a high incidence of deletions have shown that 9 to 12 exons account for 80% to 90% of all dystrophin gene deletions. The detection method uses PCR amplification of these exons, a procedure termed *multiplex PCR*.[33]

In multiplex PCR, analysis of several pairs of primers, added in the same tube concurrently, allows independent gene sequence amplification. In DMD studies, 9 to 12 primer pairs are employed simultaneously. In a normal DNA template, PCR analysis with all primer pairs should yield a specific-size product; therefore, multiplex PCR allows analysis of each of the 9 to 12 exons by using primer pairs specific to each likely exon deletion in the dystrophin gene. As with traditional PCR, electrophoretic separation and visualization reveal the presence or absence of any given deletion being evaluated. Obviously, the design of the multiplex primer set is crucial for PCR to give reliable results. A few important considerations are: (1) The primers should not have extensive complementary region; (2) the melting temperature of all the primers should be in the same range, so that a specific annealing temperature could be selected; and (3) the specific PCR products are distinguishable in size. Fig 5 shows a typical multiplex PCR-reaction gel pattern.

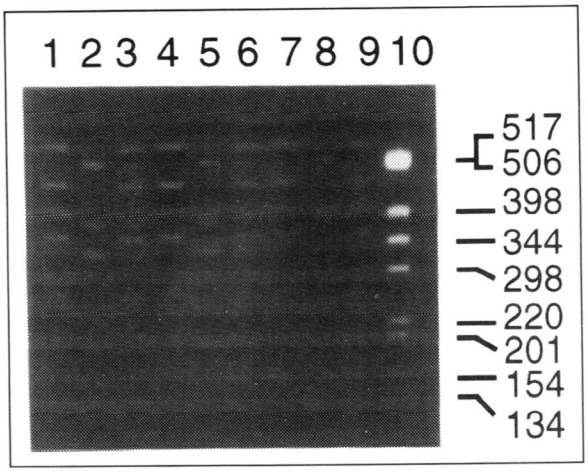

Fig 5.

Duchenne's muscular dystrophy multiplex PCR analysis. Several exons of the DMD gene are amplified in a single tube and electrophoresed.

Lane 1 represents five and lane 2 represents four different fragments amplified and resolved by agarose gel electrophoresis. Absence or mobility difference of any fragment indicates a possible deletion.

Individuals with a family history of DMD but lacking a specific, detectable deletion are tested by haplotyping and linkage analysis, using a predefined set of both intragenic and flanking DNA markers. Linkage analysis requires the evaluation of the *entire* family to satisfactorily predict the inheritance pattern of the disease.

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