MOLECULAR MEDICINE

MOLECULAR DIAGNOSIS

(First of Two Parts)

BRUCE KORF, M.D., PH.D.

MOLECULAR tools have sparked a revolution in the diagnosis of genetic disorders. In the past, genetic diagnosis was based exclusively on clinical criteria or on biochemical tests for the gene product or the consequences of its absence. Clinical criteria can be ambiguous, however, and sometimes features of an inherited disorder take years to develop, resulting in long periods of uncertainty about the diagnosis. Biochemical tests can produce equivocal results and often require invasive or expensive studies. Moreover, clinical criteria and biochemical tests have severe limitations when used to identify carriers or make a prenatal diagnosis.

Molecular methods largely avoid these problems. Such methods can, for example, unambiguously determine the presence or absence of a gene mutation in an affected person or carrier. A molecular diagnosis can be made well in advance of the appearance of clinical symptoms and can distinguish between disorders with similar phenotypes. Knowledge of the specific mutation may convey prognostic information about some disorders. Molecular diagnosis requires only a sample of DNA, which can be obtained from any source of nucleated cells, such as peripheral-blood lymphocytes or epithelial cells lining the buccal mucosa. There is no need for biopsy of affected tissue, and chorionic villus or amniotic-fluid cells can be used for a prenatal diagnosis. The high specificity of molecular diagnostic testing makes it possible, with some diseases, to screen populations for carriers.

The principal limitation in molecular diagnosis is the heterogeneity of genetic changes that underlie inherited disorders. A wide variety of changes, ranging from complete deletion of a gene to substitution of one nucleotide base in the DNA for another, can disrupt genetic function (Fig. 1). At the molecular level, some disorders are relatively homogeneous. Sickle cell disease, for example, always involves the substitution of an A for a T in the sixth codon of the β-globin gene, leading to the substitution of valine for glutamic acid as the sixth amino acid in the protein. Other disorders may involve different mutations in different people. For example, in approximately 70 percent of mutant alleles in patients with cystic fibrosis who are of northern European ancestry, a three-base deletion causes the loss of one phenylalanine from the cystic fibrosis transmembrane conductance regulator; in the other 30 percent of alleles, however, the mutations are extremely heterogeneous, with more than 200 identified so far. In other disorders, the mutations can be so diverse that almost no two affected persons have the same change. This variability hinders the design of a molecular diagnostic test that will work for all cases. In addition, some inher-

From the Genetics Division, Children's Hospital, Boston.

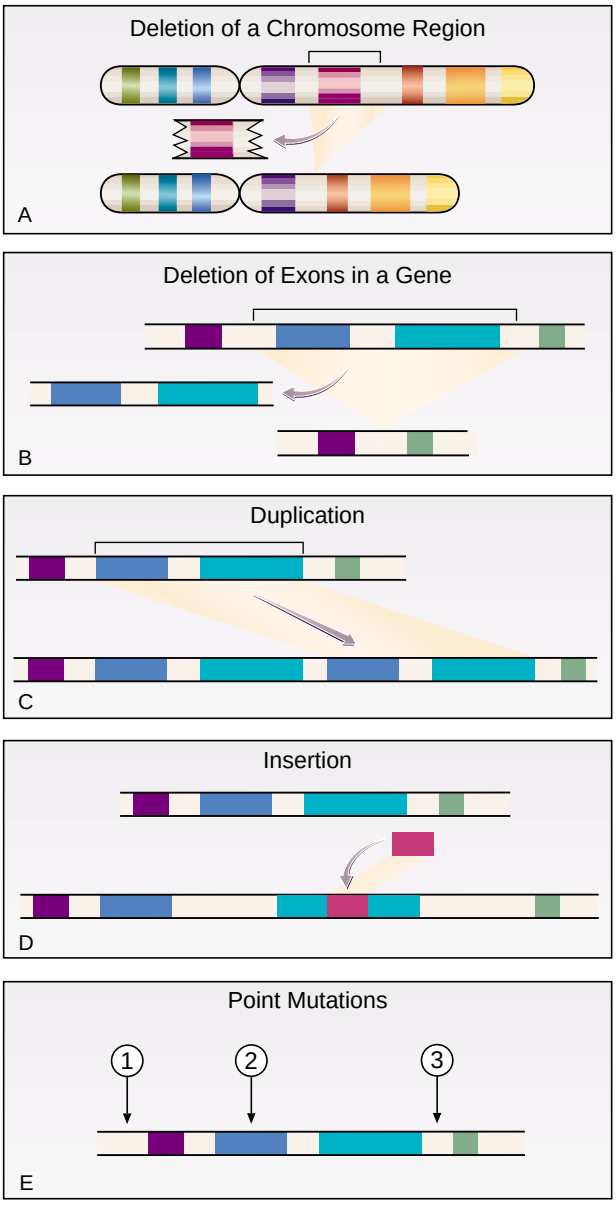

Figure 1. Types of Genetic Changes Underlying Inherited Disorders.

A chromosome region containing one or more genes may be deleted (Panel A). Several genetic disorders have been found to be due to deletions of groups of contiguous genes. If one or more exons in a gene (colored bands) are deleted (Panel B) and the newly juxtaposed exons in the final processed messenger RNA are in frame, a shortened protein will be made; if the exons are out of frame, the translation will usually be terminated prematurely because of a stop codon, resulting in a truncated protein product. Duplication of part of a gene (Panel C) leads to an increase in the size of the gene product if the exons are in frame or to truncation of the protein if the exons are out of frame. Insertion of DNA from another site in the genome (Panel D) usually leads to truncation of the protein, because a chain-termination sequence is encountered in the inserted DNA or the reading frame is altered. Point mutations, including single base changes and small insertions or deletions, can occur at various sites (Panel E). A point mutation at site 1, the promoter region of the gene, causes aberrant levels of expression. A mutation at site 2, within an exon, causes amino acid substitution or chain termination. At site 3, within an intron, a mutation alters the pattern of splicing, leading to exon skipping or the inclusion of some intron sequence in the processed messenger RNA.

ited disorders may be due to mutations in different genes in different people. For example, two distinct genes have been implicated in tuberous sclerosis in different families. Such genetic heterogeneity complicates molecular diagnosis.

The most dramatic genetic change is the deletion of an entire gene or a large part of a gene. Nevertheless, it is surprising how difficult the detection of such a gross change can be. Non–sex-linked genes come in pairs, so the normal gene tends to mask loss of all or part of the other copy. If a molecular probe for a gene is hybridized to a Southern blot of DNA from a person with a deletion of that gene, the unaffected copy of the gene will produce a normal hybridization band. The intensity of the band may be diminished because of the deletion, but quantitative analysis of a Southern blot can be difficult. Sometimes a polymorphic sequence that was inherited from only one parent can reveal the deletion of the copy inherited from the other parent.

Deletions of X-linked genes in males are easy to detect. Since a male has only a single X chromosome, loss of an X-linked sequence leaves no other representation of that sequence. Therefore, a Southern blot will show a missing band. Moreover, there will be no product of the polymerase chain reaction (PCR) for an exon that is deleted if the gene has a deleted segment (Fig. 2). The absence of PCR product or products is the basis for detecting deletions in the dystrophin gene that cause Duchenne's and Becker's muscular dystrophies. In about two thirds of males with either of these disorders there is a deletion of one or more exons in the dystrophin gene. Although the gene has 79 exons, almost all the deletions are detectable by PCR analysis of fewer than 20 exons. With PCR, groups of nine or more exons can be amplified simultaneously in the same test tube. Separation of the PCR products on an agarose gel identifies the individual exons by size. This efficient and sensitive diagnostic test takes only one day to perform. Moreover, the test sometimes provides prognostic information. Deletions that result in the severe form of muscular dystrophy, Duchenne's, tend to

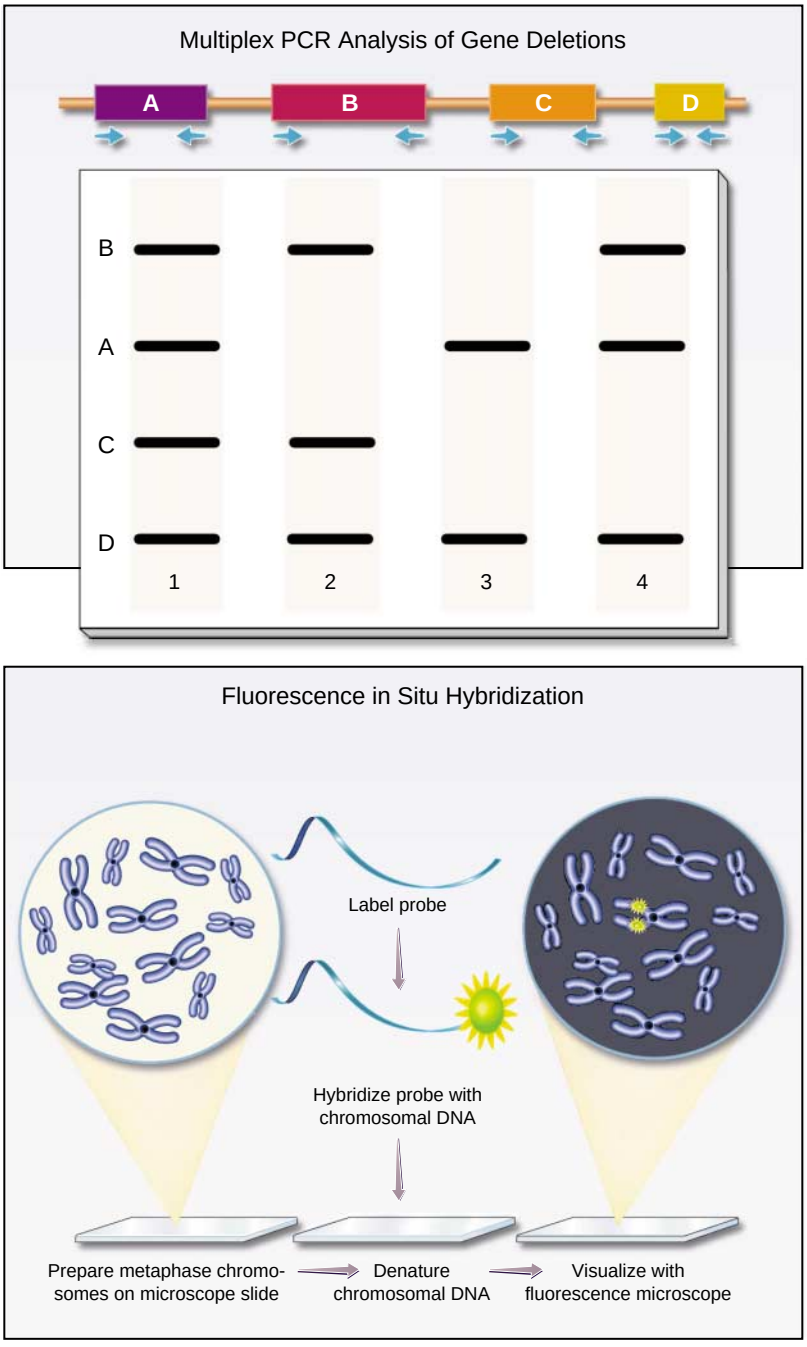

Figure 2. Two Methods of Molecular Diagnosis.

The upper panel shows multiplex PCR analysis of deletions of X-linked genes in males. Exons A, B, C, and D (colored rectangles at top) are simultaneously amplified by PCR in the same reaction tube; arrows indicate primers. The products are separated according to size on an agarose gel by electrophoresis and visualized by DNA staining. Each exon is represented by a band in the gel. If an exon is deleted, the band will be absent. Lane 1 shows a control sequence with no deletions. Deletion of one or more exons is shown in lanes 2 through 4. This is the method now used to detect deletions in the dystrophin gene causing Duchenne's or Becker's muscular dystrophy.

The lower panel shows the method of fluorescence in situ hybridization. Chromosomes in metaphase are fixed on a microscope slide, and chromosomal DNA is denatured into single strands and hybridized with a cloned piece of DNA labeled with a fluorescent marker. The chromosomal locus of the cloned DNA lights up under ultraviolet light. If there is a deletion, only one copy of the chromosome will show hybridization.

juxtapose exons in the processed messenger RNA that are not in the same reading frame. These shifts in the code — so-called frame shifts — are untranslatable into protein, and thus result in the lack of dystrophin. In the milder Becker's muscular dystrophy, the deletions juxtapose in-frame exons, resulting in a shortened, but otherwise intact, dystrophin molecule. Detection of a deletion can not only obviate the need for muscle biopsy in many cases but also allow prenatal diagnosis.

Another instance in which a deletion can be diagnostically useful is the loss of large blocks of DNA encompassing hundreds of thousands or more base pairs and, potentially, many genes. Sometimes these very large deletions can be detected by microscopical analysis of stained preparations of chromosomes in metaphase. Most of these deletions, however, are too small for light microscopy, but they can be visualized with fluorescence in situ hybridization (FISH) (Fig. 2). With this technique, a large piece of cloned DNA labeled with a fluorescent tag is hybridized to chromosomal DNA on a microscope slide. The region corresponding to the cloned DNA lights up under fluorescence illumination, unless the region has been deleted from one of the chromosomes. Several microdeletion syndromes have been discovered with the FISH method, including the Prader–Willi and Angelman syndromes (chromosome 15), the DiGeorge and velocardiofacial syndromes (chromosome 22), and aniridia with Wilms' tumor (chromosome 11). These disorders seem to represent the consequence of the simultaneous deletion of a group of genes, rather than the mutation of a single gene. In some cases, particularly in the Prader–Willi and Angelman syndromes, loss of one copy of an imprinted gene (a gene whose expression differs in the maternal and paternal copies) appears to underlie the disorder.

Other mechanisms of gross disruption of a gene include insertions of large blocks of genetic material into the substance of a gene and duplications of parts of a gene. Insertions tend to disrupt the reading frame and thus produce a truncated protein. Duplications can either increase the size of the gene product or disrupt the reading frame. About 5 percent of dystrophin mutations represent duplications. Insertions and duplications can be very difficult to detect at the molecular level.

With duplications, insertions, or deletions, a Southern blot of DNA sometimes contains an aberrant DNA fragment hybridized with cloned DNA from the mutated gene. More often, however, the rearrangement occurs at some distance from the piece recognized by the probe and thus does not appear on the Southern blot. Southern blot analysis is therefore an insensitive method of detecting the majority of these kinds of mutations.

One notable exception is the mutation of the factor VIII gene, which causes hemophilia A. About 45 percent of severely affected males have a mutation that consists of an inversion of a large segment of the gene. This phenomenon alters the pattern of hybridization of a cloned factor VIII probe on the Southern blot, thereby providing a sensitive and specific means of molecular diagnosis. It is not clear how many other genetic disorders are caused by a similar mechanism.

Most inherited conditions, however, are not due to gross deletion or rearrangement of a gene or group of genes. Instead, very small deletions or insertions of one or a few bases or single-base substitutions underlie these conditions. Detection of such mutations requires a different set of strategies, which will be described in the second part of this article.