The Use of Deoxyribonucleic Acid Probes in the Evaluation of Hemophilia

PHILLIP L. HOWARD, M.D.
American Red Cross Blood Services, Vermont New Hampshire Region and Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405

ABSTRACT

The use of deoxyribonucleic acid (DNA) probes is an important addition to existing laboratory methods for detection of carriers of hemophilia A and B. Both intragenic probes and probes directed at DNA restriction fragment length polymorphisms linked to the disease locus are now available. This technology will aid in genetic counseling of affected families and will impact upon clinical laboratory science in the near future.

Introduction

The use of DNA probes will significantly improve the accurate diagnosis of genetic disorders. The techniques have now developed to the point where their introduction into the diagnostic clinical chemistry laboratory is imminent. An important issue is the accurate identification of female carriers of X-linked recessive disorders such as hemophilia A and B. Improvement in the genetic counseling of families with hemophilia will result. This paper reviews the current literature on DNA probes available for the evaluation of hemophilia A and B.

Restriction Fragment Length Polymorphism (RFLP) and Southern Blotting

Restriction endonucleases cleave DNA in an orderly and predictable fashion based upon the presence of specific nucleotide sequences within the DNA molecule. The variable lengths of DNA fragments resulting from the action of restriction endonucleases upon DNA are called restriction fragments. In instances where specific cleavage sites exist in only some individuals, but not in others, the restriction site is said to be polymorphic. The polymorphism itself does not result in disease, since many of the polymorphic sites are located in non-expressed regions of DNA or result in amino acid substitutions which do not affect the function of the resulting protein. When the same restriction endonuclease produces different restriction fragments in different normal individuals, restriction fragment length polymorphism (RFLP) results (figure 1).

High molecular weight DNA is easily obtained from the nucleated cells in a 10 ml sample of peripheral blood collected
DNA PROBES IN HEMOPHILIA

Restriction fragment length polymorphism (RFLP). Two hypothetical strands of DNA are shown. Strand A has a restriction enzyme site (indicated by the arrow) which is absent in strand B. Following digestion by the restriction endonuclease and electrophoresis, strand A has four bands. However, because of the absence of the enzyme site in strand B, a new band appears which combines bands 3 and 4 into a new band, 7.

DNA PROBES

Two types of probes are available for the identification of specific DNA sequences in the study of genetic disorders (figure 2). The first, intragenic probes, hybridize with the actual gene under study. Intragenic probes may be genomic in that they recognize both expressed as well as non-expressed sequences within the gene or may consist of cDNA complementary to the messenger ribonucleic acid (mRNA) for the gene and thus, hybridize with only the expressed sequences of the DNA. Intragenic probe exposes a specific location on an X-ray film. The sensitivity of the method is dependent upon the specific activity of the labeled probe; however, single copy genes are easily detected. An oligonucleotide probe of as few as 14 to 20 base pairs provides a sufficiently unique sequence for specific hybridization.

DNA Probe A

Probe A

Start

Stop

Transcription

Splicing

mRNA

DNA

Probe B

FIGURE 2. Transcription of a gene. The primary RNA transcript of the gene contains both introns [-] and exons [-]. By a process of splicing out the introns, the mature messenger RNA (mRNA) transcript expresses only the genetic content of the exons. Complementary DNA (cDNA) is the DNA complementary to the mature mRNA and, hence, represents the translated portion of the gene. Probe A is extragenic in that it combines with a segment of DNA outside of but linked to the gene. Crossing over may remove an extragenic probe from its association with the gene of interest. Probe B combines with intragenic base sequences and covers both expressed and unexpressed regions. The probe need not cover the entire gene as long as it detects the desired mutant or polymorphic site.
genic probes are useful in the identification of gene deletions. Intragenic RFLP also occurs and may be used as a marker for a defective gene.

A second type of probe identifies a RFLP closely linked to the disease gene but not specifically located within the actual gene under study. In order to establish the relationship of any polymorphic restriction site to the defective gene, family studies are necessary. This is true whether the RFLP is within the gene or linked to the gene. However, an element of uncertainty enters into all linked RFLP studies owing to the potential for recombination. The lod score is used to express the degree of linkage of a RFLP to a gene. Lod scores of three or greater are considered evidence of significant linkage. In addition, polymorphism for the restriction site must exist in key family members in order for studies to be informative.

**Hemophilia B**

Hemophilia B is a sex linked recessive bleeding disorder affecting approximately one in 30,000 males. Disease results from a deficiency of coagulation factor IX. The factor IX gene is less complex than the factor VIII gene and will be discussed first. The factor IX gene is approximately 35 kb long and contains eight exons. Genomic DNA as well as complementary DNA for factor IX have been cloned by several workers. The restriction enzyme Taq I reveals a polymorphic site located 3' to exon four (figure 3). The site is detected by a portion of the genomic DNA probe (probe VIII). The Taq I RFLP should be helpful with genetic counseling of about 40% of affected families (figure 4). Additional intragenic polymorphisms with restriction enzymes Xmn I, Hinf I, and Dde I have been reported as clinically useful. In table I are summarized factor IX probes.

![Figure 3](image)

**Figure 3.** Factor IX restriction fragment length polymorphism. The probe is shown above the length of DNA which it detects. The polymorphic enzyme site (indicated by the arrow) is present within an intron of the factor IX gene. Digestion with Taq I followed by Southern blotting and probing with a genomic DNA probe reveals two bands,— one at 5.3 kb and one at 1.3 kb. Strand B, which lacks the polymorphic enzyme site, does not have the 1.3 kb band. Instead, a new band at 1.8 kb is found. Note that the 5.3 kb band is invariant. Since factor IX is X-linked, only women may be heterozygous and demonstrate both bands at 1.8 kb and 1.3 kb.

Hemophilia A

Hemophilia A is a sex linked recessive disorder affecting approximately one in 2,000 males. Disease results from a deficiency of coagulation factor VIII. The factor VIII gene is more complex than the factor IX gene and will be discussed second. The factor VIII gene is approximately 100 kb long and contains 36 exons. Genomic DNA as well as complementary DNA for factor VIII have been cloned by several workers. The restriction enzyme Xmn I reveals a polymorphic site located 3' to exon four (figure 3). The site is detected by a portion of the genomic DNA probe (probe VIII). The Xmn I RFLP should be helpful with genetic counseling of about 40% of affected families (figure 4). Additional intragenic polymorphisms with restriction enzymes Hinf I, Hinf II, and Dde I have been reported as clinically useful. In table I are summarized factor VIII probes.

![Figure 4](image)

**Figure 4.** A family with factor IX deficiency. In this hypothetical family the affected male child (III4) has inherited the 1.3 kb band from his mother and the unaffected male child (III3) had inherited the 1.8 kb band. A hemophilic grandfather (II) has passed the defective allele to the child's mother (II2). One sister (III2) is a carrier, since she exhibits the 1.3 kb band. The other sister (III1) is not a carrier.
male in 10,000. The clinical severity of bleeding correlates with the plasma level of factor VIII and the presence or absence of antibodies against factor VIII. Accurate detection of carrier females is important for genetic counseling.

The genetic linkage map of the X-chromosome has been extensively studied. Examination of an X-chromosome specific DNA library has disclosed single copy DNA sequences with RFLP linked to the factor VIII gene. The X-chromosome specific DNA probe, DX13-7, detects a Bgl II RFLP linked to factor VIII with a lod score of 5.4 (table II). Fifty percent of women are heterozygous, and the probe has proven useful in detection of female carriers of Hemophilia A. Another DNA probe designated Stl4-1 detects a Taq I RFLP linked to factor VIII with a lod score of 9.65 for which 80 percent of women are heterozygous. Additional polymorphisms have been found for the restriction enzyme Msp I. Probe Stl4-1 identifies one of the most polymorphic loci in the human genome yet discovered with at least seventeen different genotypes. The probe has proven useful in screening families for female carriers of Hemophilia A.

The factor VIII gene is the largest human gene thus far characterized. It is 186 kb in length, with 26 exons, and comprises about 0.1 percent of the entire X-chromosome. Complementary DNA copies of the 9 kb mRNA of factor VIII, as well as genomic DNA encompassing the entire factor VIII gene, have been cloned. A restriction enzyme Bcl I RFLP in exon 18 provides useful genetic information in some families (table III). Forty-two percent of females are predicted to be heterozygous for this marker. A restriction enzyme Bgl I RFLP has also been located adjacent to exon 26. The Bcl I RFLP site is most useful in people from Mediterranean countries, Asian Indians, and American blacks, 30 to 50 percent of those women being heterozygous. The Bgl I RFLP is most useful in American black women, 38 percent of whom are heterozygous.

Using the DNA probe for factor VIII, both partial deletions and nonsense point mutations have been implicated as the molecular basis for hemophilia A (table IV). In one family, an 80 kb deletion was found, and carriers could be identified from abnormal DNA located at the deletion end points.

**TABLE I**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Restriction Enzyme</th>
<th>Alleles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe VIII</td>
<td>Taq I</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Probe XII</td>
<td>Hinf I/Dde I</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>Probe VIII</td>
<td>Xmn I</td>
<td>2</td>
<td>24</td>
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**TABLE II**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Restriction Enzyme</th>
<th>Alleles</th>
<th>Lod Score</th>
<th>Reference</th>
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<tbody>
<tr>
<td>DX13-7</td>
<td>Bgl II</td>
<td>2</td>
<td>5.4</td>
<td>13</td>
</tr>
<tr>
<td>Stl4-1</td>
<td>Taq I</td>
<td>8+7</td>
<td>9.65</td>
<td>18, 19</td>
</tr>
<tr>
<td></td>
<td>Msp I</td>
<td>6</td>
<td>9.65</td>
<td>18, 19</td>
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</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Restriction Enzyme</th>
<th>Alleles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7 kb DNA (B)</td>
<td>Bcl I</td>
<td>2 + 7</td>
<td>10</td>
</tr>
<tr>
<td>1.8 kb DNA (C)</td>
<td>Bcl I</td>
<td>2 + 7</td>
<td>2</td>
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TABLE IV

Intragenic Mutation Sites Found with Factor VIII Probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Enzymes</th>
<th>Defect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7 kb cDNA (B)</td>
<td>Bel I</td>
<td>80 kb deletion (Exon 8? +22 +)</td>
<td>2</td>
</tr>
<tr>
<td>1.7 kb cDNA (A)</td>
<td>Sst I</td>
<td>80 kb deletion (Exon 8? +22 +)</td>
<td>2</td>
</tr>
<tr>
<td>9.0 kb cDNA</td>
<td>Rsa I</td>
<td>21.9 kb deletion (Exons 23 +25 +)</td>
<td>11</td>
</tr>
<tr>
<td>9.0 kb cDNA</td>
<td>Sst I</td>
<td>39 kb deletion (Exons 26 +)</td>
<td>11</td>
</tr>
<tr>
<td>4.7 kb cDNA (B)</td>
<td>Taq I</td>
<td>CGA to TGA, exon 18, Nonsense codon at amino acid 1960. Terminates synthesis.</td>
<td>2</td>
</tr>
<tr>
<td>4.7 kb cDNA (B)</td>
<td>Hind III</td>
<td>Point mutation or, rare polymorphism. Uncertain mechanism of disease.</td>
<td>2</td>
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<tr>
<td>9.0 kb cDNA</td>
<td>Taq I</td>
<td>CGA to TGA in exon 24. An inframe stop codon 24 amino acids too short.</td>
<td>11</td>
</tr>
<tr>
<td>cDNA</td>
<td>Taq I</td>
<td>CGA to TGA in exon 26. An inframe stop codon 26 amino acids too short.</td>
<td>11</td>
</tr>
<tr>
<td>cDNA</td>
<td>Taq I</td>
<td>CGA to TGA in intron 2. Uncertain mechanism of disease.</td>
<td>11</td>
</tr>
<tr>
<td>9.0 kb cDNA</td>
<td>Taq I</td>
<td>CAA to CGA in intron 25 creates a new Taq I site. Uncertain mechanism of disease.</td>
<td>11</td>
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</tbody>
</table>

**Summary**

There is significant promise that DNA probe technology can improve genetic counseling for a variety of inherited disorders. There are, however, some important scientific and practical limitations. For X-linked inheritance, women must be heterozygous at the disease locus gene for studies to be informative. For extragenic linked polymorphisms, the results continue to carry a level of uncertainty owing to the possibility of recombination. Moreover, key family members need to be available for testing. Finally, DNA probe technology is an adjunct to existing laboratory methods for carrier testing in Hemophilia. It should not be considered a replacement for existing methods.

Few probes are commercially available. Most probes must now be obtained from colleagues who are willing to share them for purposes of research or genetic counselling. Commonly, restrictions are placed on the use and distribution of shared probes. As with any promising and emerging technology, the interest of manufacturers is keen. The DNA probe technology can probably be compared to radioimmunoassay of 20 years ago. In the early days of radioimmunoassay, many laboratories made their own antibodies. Today, most laboratories purchase RIA reagents in the form of kits.

**Glossary of Special Topics**

*Complementary DNA (cDNA)—A sequence of DNA copied from RNA by reverse transcription.*

*Exon—Sequences of genomic DNA which are transcribed into messenger RNA and ultimately expressed or translated into proteins.*

*Genomic DNA—Chromosomal DNA as it occurs in a gene including both introns and exons.*

*Intron—Sequences of genomic DNA which are not transcribed into messenger RNA and are thus untranslated. Also called intervening or untranslated sequences.*

*Kilobase (kb)—One thousand nucleotide bases.*

*Lod Score—The likelihood of linkage between two loci. Lod is shorthand for the “logarithm of the odds” that the loci are linked. If the odds favoring linkage are a thousand to one, the lod is 3. A lod of 3 or greater is usually considered significant evidence of linking.*
**Probe**—Sequences of single stranded nucleic acids which are complementary to RNA or DNA and thus will specifically hybridize. For purposes of identifying the complementary DNA or RNA the probe is labeled either with a radionuclide or an enzyme.

**Restriction Endonuclease**—Enzymes which break the phosphodiester bonds between specific nucleotides. Many of these enzymes recognize sequences of four or six nucleotides. The enzymes are derived from bacteria and are named after the parent organism and the order of isolation. Thus the enzyme, EcoRI, is the first restriction endonuclease derived from *Escherichia coli*.

**References**


