Molecular Defect in Factor IX_Hill, a Hemophilia Bm Variant: Arg → Gln at the Carboxyterminal Cleavage Site of the Activation Peptide

By Min-Ning Huang, Carol K. Kasper, Harold R. Roberts, Darrel W. Stafford, and Katherine A. High

A genomic DNA library and the enzymatic DNA amplification technique were used to isolate unique factor IX coding sequences of a hemophilia Bm variant, factor IX_Hill. A point mutation that resulted in the substitution of a glutamine (CAG) for an arginine (CGG) at amino acid 180 was found in exon VI of the factor IX gene (G ←→ A at nucleotide 20518). This mutation alters the carboxy terminal cleavage site for the activation peptide at Arg<sup>180</sup>-Val<sup>181</sup>. The arginine residue at the activation peptide cleavage site is conserved in mouse, canine, bovine, and human factor IX, suggesting that the arginine at amino acid 180 is important for normal cleavage. Sequencing of all of the coding regions of factor IX_Hill revealed no other mutations. We have also shown that the point mutation in exon VI creates a new Dde I restriction site, which, in combination with the enzymatic DNA amplification technique, provides a quick, reliable, and sensitive method for carrier detection and antenatal diagnosis in affected kindreds. This is the first report of the molecular defect in a hemophilia Bm patient with a markedly prolonged ox brain prothrombin time.© 1989 by Grune & Stratton, Inc.

**MATERIALS AND METHODS**

**Patient profile.** The patient studied is an Asian kindred (Americans of Japanese descent) with severe hemophilia B. The factor IX activity level is <1%, and the antigen level is 120%. The ox brain prothrombin time, performed using the method of Kasper et al.,<sup>6</sup> is markedly prolonged at 173 seconds (control 40.9 seconds).

**Isolation and characterization of gene encoding factor IX<sub>Ilm</sub>.** The strategy for the characterization of the hemophilic factor IX gene involved isolating and sequencing all of the coding regions, exon-intron junctions, and most of the 3' untranslated region. A genomic library was constructed and screened to recover exons II through V and VII, VIII. Exons I and VI were isolated using an enzymatic amplification technique.<sup>11</sup>

**Exons II through V, VII, and VIII all occur on 5.0 to 5.5 kb EcoRI fragments of the factor IX gene. Thus a size-selected fraction (5.0 to 5.5 kb) of EcoRI-digested patient DNA was used to construct a genomic library. High mol wt DNA was isolated from peripheral blood using standard procedures.<sup>12</sup> DNA was digested to completion with EcoRI (New England Biolabs, Beverly, MA) according to manufacturer’s recommendations and electrophoresed on a 0.8% agarose gel. The 5.0 to 5.5 kb fraction was collected and ligated into the EcoRI site of the vector pGEM-3Z (Promega Biotech, Madison, WI) in the presence of the lac gene of pBR322. The libraries were not amplified. Screening of duplicate filter lifts from the size-selected libraries was carried out by the protocol of Benton and Davis<sup>14</sup> using nick-translated probes. The

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FAULT IX-HEMOPHILIA B

Table 1. Primers Used for Enzymatic Amplification for Exon I and Exon VI and Their Locations in the Human Factor IX Gene*

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Size bp</th>
<th>Sequence (5' (\rightarrow) 3')</th>
<th>Location</th>
<th>Position With Respect to Corresponding Exon</th>
<th>Size of Amplified Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A1</td>
<td>31</td>
<td>CTCAAGAATTCCACAGTGGAGAGCCCACGG</td>
<td>-188 to -158</td>
<td>188 bp upstream</td>
<td>467 bp</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>31</td>
<td>AGAAAGGAAATTCCTAACCACCTGGTCTGAGGC</td>
<td>270 to 240</td>
<td>162 bp downstream</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>F1</td>
<td>31</td>
<td>TTTTGAATTCTGATGGGGCTCCTTCTCAG</td>
<td>20235 to 20265</td>
<td>128 bp upstream</td>
<td>397 bp</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>31</td>
<td>TCAGTGAATTCCTGGCTGTCTGTCAG</td>
<td>20634 to 20604</td>
<td>69 bp downstream</td>
<td></td>
</tr>
</tbody>
</table>

*The underlined sequences were altered from the original to create EcoRI sites for subcloning. The primers for Exon VI were also used in the Ddel analysis.

†Numbers refer to sequence published in reference 1.

RESULTS

Three different clones, which included six of the eight exons, were isolated from the genomic library. Restriction enzyme analysis of these clones revealed that all the EcoRI fragments were of the size predicted by the published restriction map of factor IX, making a gene deletion or insertion in any of these unlikely. Repeated screening of appropriate size-selected genomic libraries for exons I and VI failed to yield any positive plaques. Fragments containing exon I (117 bp) and exon VI (203 bp) and approximately 100 bp of intron on either side of the exon were therefore isolated using the enzymatic amplification technique.

Nucleotide sequence for all of the exons, exon-intron junctions, and 1271 bp of noncoding region was determined (Table 2). Approximately 60% of the coding regions, including the fragment containing the mutation, were sequenced in both orientations. Sequence analysis revealed two differences between the factor IXHb sequence and the published normal sequence. One difference, at residue 31603 (numbered according to reference 1), consisted of the deletion of a single cytosine in the 3' untranslated region, 234 bp downstream from the stop codon, and more than 1,000 bp upstream from the pol(A) signal and the pol(A) addition site. It is also distant from the putative hairpin structure in the 3' untranslated region. Although the sequence varies from that reported by Yoshitake et al., it is identical to that reported by Jave et al for the factor IX cDNA. Thus it seems unlikely that this difference, if it does indeed represent a variation from normal, could account for the hemophilic phenotype. The second mutation occurs within the coding region at exon VI at the codon for amino acid 180. Figure 1 displays the DNA coding sequence of factor IXHb and normal factor IX along with their respective translation products. In factor IXHb, nucleotide 20519 (numbered according to reference 1) is changed from the guanine to an adenine. This results in the substitution of a glutamine (CAG) for an arginine (CGG) at amino acid 180. Since this amino acid is at the cleavage site for the activation peptide, one would predict that a substitution here could result in a protein that could not be cleaved or that was cleaved slowly by factor XIa (a IXa variant). Analysis of factor IX protein isolated from this patient's plasma confirms that the protein is cleaved at a very slow rate by factor XIa at the Arg-GluVal bond (McCord D, Monroe H, Huang MN, Kasper CK, High KA, Roberts HR, unpublished observations).
The substitution of an A for a G at residue 20519 creates a new restriction enzyme recognition site for the enzyme \textit{DdeI}. \textit{DdeI} recognizes and cleaves the sequence CTNAG (where N is any nucleotide). This sequence is present in factor IX$^{\text{Hilb}}$ but not in normals (Fig 2). Enzymatic amplification of DNA from a normal control and subsequent digestion with \textit{DdeI} results in the pattern shown in lane 1, with bands of 182 bp and 141 bp (and several smaller fragments as indicated on the map). The same analysis for factor IX$^{\text{Hilb}}$ (lane 2) results in fragments of 141, 99, and 83 bp, the 182 bp fragment being reduced to the two smaller fragments following cleavage at the newly created restriction site.

\section*{DISCUSSION}

Hemophilia B$^m$ was initially described by Hougie and Twomey in 1967\textsuperscript{7} and is characterized by a prolongation of the PT in the presence of bovine tissue factor, in addition to the prolongation of the activated PTT that is typical for hemophilia B. Through characterization of individual B$^m$ variants,\textsuperscript{8,9} evidence has accumulated that they comprise a heterogeneous group of disorders. For example, a variant described by Osterud et al\textsuperscript{8} as well as one described by Usharani et al\textsuperscript{9} undergo normal proteolysis by XIa-Ca$^{+2}$, whereas factor IX Deventer, another B$^m$ variant, cannot be cleaved at the Arg$^{180}$-Val$^{181}$ bond.\textsuperscript{8} Failure to cleave at Arg$^{180}$-Val$^{181}$ results in a molecule (termed factor IX$^\alpha$) with no detectable clotting activity.

The conservation across species of the Arg-Val cleavage site of the activation peptide attests to the critical nature of these residues in factor IX function. Mouse (Wu S, Ware J, Stafford DW, unpublished results), canine (Evans JP, Watzke HH, Ware JL, Stafford DW, High KA, unpublished results), bovine,\textsuperscript{2} and human\textsuperscript{1} factor IX all possess Arg-Val at the carboxyterminal cleavage site of the activation peptide. Cleavage sites at the activation peptide in other human vitamin-K dependent procoagulant proteins (II, VII, X) also exhibit this conservation of the arginine residue, although the valine is not conserved. The conservation of arginine at the carboxyterminal cleavage site of the activation peptide suggests that it is important for normal factor-IX function. One would predict that a mutation resulting in a change at Arg$^{180}$ would result in a form that could not be cleaved normally; indeed this has been demonstrated for factor IX$^{\text{Hilb}}$ (McCord D et al, unpublished results), a newly described factor IX$^\alpha$ variant.

How the failure to cleave at Arg$^{180}$-Val$^{181}$ results in a prolongation of the ox-brain PT remains unexplained. It is interesting to note however, that factor IX Deventer, also a B$^m$ variant with an ox-brain PT approximately three times normal; indeed this has been demonstrated for factor IX$^{\text{Hilb}}$ (McCord D et al, unpublished results), a newly described factor IX$^\alpha$ variant.
The creation of a new restriction site in the mutant allele provides a quick, simple, and reliable means for screening for the presence of the mutation. Carrier detection based on protein levels in hemophilia is often indeterminate, since normal and carrier levels may overlap. RFLP analysis for antenatal diagnosis and carrier detection allows determination of carrier status in approximately 70% of families, but this type of analysis is relatively tedious, since it may require the study of a large number of family members to establish the pattern of inheritance of a given polymorphism with a particular hemophilic gene. In the case of factor IX, the presence of a new restriction site combined with the enzymatic amplification technique provides a rapid (<24 hours) and sensitive (requires only 1 μg of patient DNA) means for identifying the mutant allele. This restriction site should prove useful both in carrier detection and antenatal diagnosis in affected kindreds and as a means for screening other B variants to ensure that resources are not committed to sequencing a mutation already characterized.

ACKNOWLEDGMENT

The authors thank Dr Jerry Ware for valuable discussion during this project. The authors acknowledge the skillful secretarial assistance of Dianne Partin.

ADDENDUM

After this manuscript was submitted, Spitzer et al22 reported a different mutation, at amino acid 390, in a patient with a markedly prolonged ox brain prothrombin time.

REFERENCES

Molecular defect in factor IXHilo, a hemophilia Bm variant: Arg----Gln at the carboxyterminal cleavage site of the activation peptide

MN Huang, CK Kasper, HR Roberts, DW Stafford and KA High