Molecular Defect in Factor IX<sub>HblO</sub>, a Hemophilia B<sub>m</sub> Variant: Arg → Gln at the Carboxyterminal Cleavage Site of the Activation Peptide

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Factor IX is a vitamin-K dependent clotting factor required for normal hemostasis; a deficiency of functional factor IX results in the X-linked bleeding diathesis hemophilia B. Factor IX is synthesized in the liver as a single-chain polypeptide precursor of 461 amino acids. The protein subsequently undergoes a number of post-translational modifications required for normal activity, including vitamin-K dependent carboxylation of the first 12 glutamic acid residues and removal of the 46 residue signal peptide sequence. The mature protein of 415 aa circulates as a zymogen; it can be activated either by factor XIa, in a reaction requiring Ca<sup>2+</sup>, or by factor VIIa-tissue factor, also requiring Ca<sup>2+</sup>. Activation by either of these enzymes involves cleavage of two bonds, Arg<sup>180</sup>-Val<sup>181</sup>, in factor IX to yield a disulfide-linked serine protease, factor IXa, and a 35 amino-acid activation peptide. Factor IXa then proteolytically converts factor X to Xa in a reaction requiring Ca<sup>2+</sup>, phospholipid, and factor VIIIa.

Factor IX-deficient patients are a heterogeneous group both clinically and at the molecular level. A number of classifications have proven useful in distinguishing among subtypes. One of the most widely used, for example, subdivides patients on the basis of whether factor IX antigen (crossreacting material) can be detected in the plasma (CRM-positive, CRM-reduced, CRM-negative). Functional assays have also been used; one assay that has proved useful for defining a distinct subgroup of CRM positive hemophilia B patients is the ox-brain prothrombin time. In most cases patients with hemophilia B have a prolonged partial thromboplastin time (PTT) and a normal prothrombin time (PT). In 1967 Hougie and Twomey described two brothers who had markedly prolonged (>3 × normal) prothrombin times when ox brain was used as the source of tissue factor. The defect was designated hemophilia B<sub>m</sub>, the m referring to the family name of the patients. Since then a number of other kindreds have been shown to have a similar defect, although on the basis of other functional assays it appears that these B<sub>m</sub> variants differ from each other. In this paper we report the identification, using complete DNA sequence analysis of the coding regions of the variant IX, of the mutation responsible for the hemophilia B<sub>m</sub> variant. We have predicted the amino acid substitution and the functional characteristics of the variant protein. In addition, we have demonstrated a quick and reliable method for screening for the presence of the mutant allele; this should facilitate antenatal diagnosis and carrier detection in kindreds with this defect.

MATERIALS AND METHODS

Patient profile. The patient studied is from 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, is markedly prolonged at 173 seconds (control 40.9 seconds).

Isolation and characterization of gene encoding factor IX<sub>Bm</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.

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. 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 Act Wex-A<sub>B</sub>. Commercially available packaging extracts (Promega Biotech, Madison, WI) were used, and 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 using nick-translated probes. The
probes used included plasmid subclones of the factor IX Alabama gene and the human factor IX cDNA. Phage DNA was prepared, subcloned into M13 sequencing vectors, and sequenced as previously described by Ware et al. Sequence data were analyzed using Beckman Microgenie sequencing programs.

Exons I and VI were isolated using the enzymatic amplification technique. Thirty-one base synthetic oligonucleotides, flanking exons I and VI and containing EcoRI restriction sites, were used to prime amplification (Table 1). Target sequences were amplified in a 100 μL volume containing 1 μg of genomic DNA, 16.6 mmol/L (NH₄)₂SO₄, 67 mmol/L Tris-HCl, pH 8.8, 6.7 mmol/L MgCl₂, 6.7 mmol/L Na₂ EDTA, 10 mmol/L BME, 10% dimethyl sulfoxide (DMSO), 1.5 mmol/L each of deoxyribonucleoside triphosphates (dNTP: dATP, dCTP, dTTP, dGTP), 1 μmol/L of each primer and 2 units of Taq polymerase (obtained from Perkin-Elmer, Norwalk, CT). The samples were overlaid with 100 μL of mineral oil to prevent condensation and subjected to 25 cycles consisting of a three-minute denaturation period at 94°C, a three-minute annealing period at 70°C, and a five-minute extension period at 72°C. Following amplification the DNA was electrophoresed on a 3.0% agarose gel and stained with ethidium bromide for visual detection of the amplified products. The samples were then extracted from the gel as outlined by Maniatis et al. The amplified DNA was digested with EcoRI, subcloned into an M13 vector, and sequenced as described above. To minimize the possibility of errors due to Taq polymerase, fragments isolated from two separate amplification reactions were sequenced in both orientations; all of these yielded identical results.

The DdeI restriction analysis, which identifies the mutant allele, was carried out according to manufacturer’s directions. Substrates for the analyses were amplified patient and control DNA.

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 IX₉₉ sequence and the published normal sequence. One difference, at residue 31603 (numbered according to reference 1), consisted of the deletion of a single cysteine in the 3’ untranslated region, 234 bp downstream from the stop codon, and more than 1,000 bp upstream from the polya signal and the polya 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 Jaye 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 IX₉₉ and normal factor IX along with their respective translation products. In factor IX₉₉ 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 Xa (a Xa 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 Xa at the Arg₁₈₀ Val₁ₘ bond (McCord D, Monroe H, Huang MN, Kasper CK, High KA, Roberts HR, unpublished observations).

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>Primer</th>
<th>Name</th>
<th>Size (bp)</th>
<th>Sequence (5'→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>CTCAAGAATTCCACAGTGCGCAGAGCCACAG</td>
<td>−188~−158</td>
<td>188 bp upstream</td>
<td>467 bp</td>
</tr>
<tr>
<td></td>
<td>A₂</td>
<td>31</td>
<td>AGAACGAAATCTTACAACTCTGCTGCTGTCGTC</td>
<td>270~240</td>
<td>162 bp downstream</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>F₁</td>
<td>31</td>
<td>TTTTTGATTCCTGATGGGCCCTGCTTTCAG</td>
<td>20235~20265</td>
<td>128 bp upstream</td>
<td>397 bp</td>
</tr>
<tr>
<td></td>
<td>F₂</td>
<td>31</td>
<td>TCAGTGAAATCCCTGCTGTTGCTTGCCAG</td>
<td>20634~20604</td>
<td>69 bp downstream</td>
<td></td>
</tr>
</tbody>
</table>

*The underlined sequences were altered from the original to create EcoR I sites for subcloning. The primers for Exon VI were also used in the DdeI analysis.
†Numbers refer to sequence published in reference 1.

Table 2. Length of DNA Sequences Determined for the Gene of Factor IX₉₉

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence</th>
<th>Nucleotides Identical to Normal F.IX*a</th>
<th>Nucleotides 5' to Exon</th>
<th>Coding Sequence</th>
<th>Nucleotides 3' to Exon</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>182</td>
<td>182/182</td>
<td>33</td>
<td>117f</td>
<td>32</td>
</tr>
<tr>
<td>II</td>
<td>99</td>
<td>99/99</td>
<td>35</td>
<td>164</td>
<td>22</td>
</tr>
<tr>
<td>III</td>
<td>187</td>
<td>187/187</td>
<td>115</td>
<td>25</td>
<td>47</td>
</tr>
<tr>
<td>IV</td>
<td>160</td>
<td>160/160</td>
<td>11</td>
<td>114</td>
<td>34</td>
</tr>
<tr>
<td>V</td>
<td>301</td>
<td>301/301</td>
<td>142</td>
<td>128</td>
<td>30</td>
</tr>
<tr>
<td>VI</td>
<td>357</td>
<td>356/357</td>
<td>58</td>
<td>203</td>
<td>96</td>
</tr>
<tr>
<td>VII</td>
<td>153</td>
<td>153/153</td>
<td>26</td>
<td>115</td>
<td>12</td>
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<tr>
<td>VIII</td>
<td>1109</td>
<td>1108/1109</td>
<td>69</td>
<td>545</td>
<td>609‡</td>
</tr>
</tbody>
</table>

*aUsing the published sequence of Yoshitake et al.
†Includes 29 nucleotides of 5’ untranslated region.
‡Nucleotides starting at the TAA stop codon and proceeding into the 3’ untranslated portion of exon VIII.
The substitution of an A for a G at residue 20519 creates a new restriction enzyme recognition site for the enzyme Ddel. Ddel recognizes and cleaves the sequence CTNAG (where N is any nucleotide). This sequence is present in factor IXhio, but not in normals (Fig 2). Enzymatic amplification of DNA 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 IXhio (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.

**DISCUSSION**

Hemophilia Bm was initially described by Hougie and Twomey in 1967 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 Bm variants, evidence has accumulated that they comprise a heterogeneous group of disorders. For example, a variant described by Osterud et al as well as one described by Usharani et al undergo normal proteolysis by XIa-Ca++, whereas factor IX Deventer, another Bm variant, cannot be cleaved at the Arg180-Val181 bond. Failure to cleave at Arg180-Val181 results in a molecule (termed factor IXα) 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, and human 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 sug-

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**Fig 1.** Sequence of the region of the human factor IX gene coding for exon VI of factor IXhio and of normal factor IX, along with their respective translation products. The numbers indicate the positions of corresponding amino acids in the protein. Arrow indicates the point mutation. The change from a guanine to an adenine in factor IXhio results in the substitution of glutamine (CAG) for arginine (CGG) at amino acid 180.

**Fig 2.** Ddel restriction digest of amplified DNA fragment including exon VI on 12% polyacrylamide gel. M, marker: Lane 1, normal DNA pattern with two bands, 182 and 141 bp; Lane 2, factor IXhio, with three bands in which the 182 bp band has disappeared and is replaced with two smaller bands, 99 and 83 bp. The restriction map below the gel indicates Ddel restriction sites within the amplified DNA fragment and the origin of the bands seen in the gel. The mutation in factor IXhio creates a new restriction site for Ddel (CTCAG) as indicated. Note that this Ddel restriction site is distinct from the Ddel polymorphic site flanking exon II, previously reported to have utility in carrier detection.
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

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ADDENDUM

After this manuscript was submitted, Spitzer et al. 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