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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A genomic DNA library and the enzymatic DNA amplification technique were used to isolate human factor IX coding sequences of a hemophilia B<sub>m</sub> variant, factor IX<sub>HblO</sub>. 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 20519). 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<sub>HblO</sub> 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 B<sub>m</sub> patient with a markedly prolonged ox brain prothrombin time.

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proxies 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 Na₂EDTA, 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 annealing period at 55°C, and four minutes at 70°C to extend the annealed primers. Following amplification the DNA was electrophoresed on a 3.0% agarose gel in Tris-borate-EDTA buffer. DNA from the amplified region was 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 IXHBo 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 polynucleotide signal and the poly(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 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 IXHBo and normal factor IX along with their respective translation products. In factor IXHBo, 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 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 Xa at the Arg240Val241 bond (McCord M, 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>Exon</th>
<th>Primer</th>
<th>Size</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>CTCAAGAATCCACAGTGCGAGAAGCCCACG</td>
<td>-188 ~ -158</td>
<td>188 bp upstream</td>
<td>467 bp</td>
</tr>
<tr>
<td>I</td>
<td>A₂</td>
<td>31</td>
<td>AGAAAGAATTTCTACGAACCTGGTCCTGGC</td>
<td>-270 ~ -240</td>
<td>162 bp downstream</td>
<td>467 bp</td>
</tr>
<tr>
<td>VI</td>
<td>F₁</td>
<td>31</td>
<td>TTTTTTGTACTGTGGGCTCTGTCTTCAG</td>
<td>20235 ~ 20265</td>
<td>128 bp upstream</td>
<td>397 bp</td>
</tr>
<tr>
<td>VI</td>
<td>F₂</td>
<td>31</td>
<td>TCAGTGAACTCGCTCTGTCGTCGTCCAG</td>
<td>20634 ~ 20604</td>
<td>69 bp downstream</td>
<td>397 bp</td>
</tr>
</tbody>
</table>

*The underlined sequences were altered from the original to create EcoR1 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 IXₕBo

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence</th>
<th>Nucleotides Identical to Normal F. IX*</th>
<th>Nucleotides 5' to Exon</th>
<th>Coding Sequence Nucleotides 3' to Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>182</td>
<td>182/182</td>
<td>33</td>
<td>117†</td>
</tr>
<tr>
<td>II</td>
<td>99</td>
<td>99/99</td>
<td>35</td>
<td>164‡</td>
</tr>
<tr>
<td>III</td>
<td>187</td>
<td>187/187</td>
<td>115</td>
<td>25‡</td>
</tr>
<tr>
<td>IV</td>
<td>160</td>
<td>160/160</td>
<td>11</td>
<td>114‡</td>
</tr>
<tr>
<td>V</td>
<td>301</td>
<td>301/301</td>
<td>142</td>
<td>129‡</td>
</tr>
<tr>
<td>VI</td>
<td>357</td>
<td>356/357</td>
<td>58</td>
<td>203‡</td>
</tr>
<tr>
<td>VII</td>
<td>153</td>
<td>153/153</td>
<td>26</td>
<td>115‡</td>
</tr>
<tr>
<td>VIII</td>
<td>1109</td>
<td>1108/1109</td>
<td>69</td>
<td>545‡</td>
</tr>
</tbody>
</table>

*Using 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 DdeI. DdeI recognizes and cleaves the sequence CTNAG (where N is any nucleotide). This sequence is present in factor IXb, but not in normals (Fig 2). Enzymatic amplification of DNA from a normal control and subsequent digestion with 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 IXb (lane 2) results in a new restriction site for the enzyme DdeI. The restriction map below the gel indicates that this restriction site is distinct from the restriction site for DdeI (CTCAG) as indicated. Note that this DdeI restriction site is distinct from the DdeI polymorphic site flanking exon II, previously reported to have utility in carrier detection.

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 Xla-Ca++, whereas factor IX Deventer, another Bm variant, cannot be cleaved at the Arg80-Val81 bond. Failure to cleave at Arg80-Val81 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 sites 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 Arg180 would result in a form that could not be cleaved normally; indeed this has been demonstrated for factor IXb (McCord D et al, unpublished results), a newly described factor IXα variant.

How the failure to cleave at Arg180-Val181 results in a prolongation of the ox-brain PT remains unexplained. It is interesting to note however, that factor IX Deventer, also a Bm variant with an ox-brain PT approximately three times that of normals (Fig 2), has been reported to have a mutation at Arg180, in this case an arginine to a tryptophan (CGG → TGG), previously reported to have utility in carrier detection.

Fig 1. Sequence of the region of the human factor IX gene coding for exon VI of factor IX, 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 IX results in the substitution of glutamine (CAG) for arginine (CGG) at amino acid 180.
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.

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ADDENDUM

After this manuscript was submitted, Spitzer et al24 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