CONCISE REPORT

Hemophilia B Durham: A Mutation in the First EGF-Like Domain of Factor IX That Is Characterized by Polymerase Chain Reaction

By Peter H. Denton, Dana M. Fowlkes, Susan T. Lord, and Howard M. Reisner

Two men with factor IX (FIX) antigen-positive (CRM+) hemophilia B were selected for study because of their abnormal expression of an immunologically defined epitope previously localized to the EGF-like domains of the molecule. Exons IV and V (coding for the first and second EGF-like domains) of FIX were amplified 10^4 times from the patients’ genomic DNA by using polymerase chain reaction (PCR) technology and sequenced. Both patients had identifiable mutations which resulted in the highly conserved Gly 60 residue being changed to Ser. PCR-amplified exon IV from six normal males had the previously defined canonical sequence. The correlation between the mutation and defective epitope expression in the two patients suggests that a change in the tertiary structure of the EGF-like domain is likely to cause the mild hemophilia B.

© 1988 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Patients: Patient 1 is a 55-year-old male recently diagnosed as having mild hemophilia B (FIX:C, 14%). The patient had a history of prolonged bleeding following dental extraction but has not required replacement therapy. His bleeding tendency has been lifelong, and he had a similarly affected maternal uncle. Patient 2 is a 34-year-old male whose hemophilia was first diagnosed at the age of 10 following episodes of posttraumatic hematoma formation and prolonged bleeding after minor dental and surgical procedures. Mild hemophilia B (FIX:C, 9% to 16%) was diagnosed at the age of 27 following an upper gastrointestinal bleed that required replacement therapy. Limited pedigree data suggest that the two patients are not related.

DNA extraction. Nuclear pellets were obtained from whole blood and extracted by using a proprietary DNA purification system (The Extractor, Molecular Biosystems, Inc, San Diego).

Oligonucleotide primers. Automated synthesis of primers was carried out with an Applied Biosystems (Foster City, CA) 380-A synthesizer using reagents from American BioMicro (Hayward, CA). Primer purification was by trityl reverse-phase high-performance liquid chromatography. The following primers were used (bases in lowercase indicate introduced restriction sites): (a) exon 4 (5') end, BamiI/1 5'CATCCggtccgtcATGTATCGGGAGAGCAGGGCC; (b) exon 4 (3') end, Sac I 5'CAACTTgggctcGACCCCAATTGAG; (c) exon 5 (5') end, BamHI/1 5'CAATGGTggtccgACCCATCACTAGTACATCCTGACGTT; (d) exon 5 (3') end, Sac I 5'TTCTAagctctAAAAAGAAGGATCTAAAGTCAAGTCCAAGTACGG; (e) exon 4 forward sequencing, 5'CGTGCACATTCAATTCTTACC; and (f) exon 4 reverse sequencing, 5'GGGAAAATTGAACCATGAG.

PCR. The reaction was done in glycerine-filled heating blocks regulated by external controllers (type RTD, Omega Electronics, Stamford, CT). The method was that of Kogan et al^1^ with a modified temperature cycle of three minutes at 94°C, three minutes at 55°C, and four minutes at 70°C. Taq polymerase was added in a single aliquot (3 units, Cetus Corp, Emeryville, CA) at the end of the initial denaturation. The mixture was overlaid with mineral oil to minimize evaporation. After 30 cycles the reaction mixture was ethanol precipitated, the DNA redissolved, and electrophoresed on a 4% Nusieve agarose gel. Bands of interest were excised and the DNA recovered by electroelution (IBI [New Haven, CT] UEA electrophoreser using 1/4 × TBE and a 12 mol/L ammonium acetate salt cushion at 75 V).

Cloning and sequencing of amplified regions. The restricted PCR fragments were cloned into pBS (+/−) (Stratagene, Inc, La Jolla, CA), and plasmid “minipreps” were prepared before double-stranded plasmid sequencing.® For plasmid sequencing 1 μg of.

From the Departments of Microbiology and Immunology and Pathology, University of North Carolina at Chapel Hill.

Submitted April 29, 1988; accepted June 10, 1988.

Supported in part by Grant HL-06350 from the National Heart, Lung, and Blood Institute.

Address reprint requests to Howard M. Reisner, PhD, Department of Pathology, CB 7525, Rm 622 BBB 228-H, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc. 0006-4971/88/7204-0026$3.00/0
DNA was made 0.2 N in NaOH and 0.2 mmol/L in EDTA and brought to 42°C for 15 minutes and ethanol precipitated. Sequencing was done by the Sequenase method (USB Biochemicals, Cleveland) except that the termination mixes were (a) 0.3 mmol/L, 0.1 mmol/L, 0.15 mmol/L, or 0.5 mmol/L in their respective dideoxy A, C, G, or T; (b) 0.025 mol/L in the corresponding deoxy A, C, G, or T; and (c) 0.25 mmol/L in the other three deoxynucleotides. Direct sequencing on the amplified DNA was performed as before except that 300 to 500 ng of PCR fragment was denatured at 95°C for five minutes and then annealed in the presence of primer at 37°C for five minutes before sequencing.

RESULTS

By using the modified PCR and primers a to d as indicated earlier, a minimum 105-fold amplification was routinely achieved for exons 4 and 5 of all FIX genes studied. Primers were chosen so as to lie about 100 base pairs (bp) on either side of the splice junctions of the target exons. The primer location resulted in a major amplified product having a length consistent with that of 252 bp for exon 4 and 341 bp for exon 5 as expected. The product of PCRs when using the two mutant or 20 normal DNAs were indistinguishable on agarose gels. The resulting yield of 1 or more μg of amplified exonic fragment was sufficient for two direct sequencing reactions or ten clonal ligations.

To identify the mutation in exon 4 or 5 of the patients' DNA, the amplified regions were independently cloned. In both patients a G-to-A transition was found by ds-DNA plasmid sequencing at nucleotide 10,430 in exon 4 (numbering as by Yoshitake et al). This was confirmed by sequencing the opposite plasmid strand (for both patients) as well as sequencing a second independently cloned PCR fragment (for one patient). Thus a glycine (GGC) at amino acid 60 is changed to a serine (AGC) in the FIX of both patients (Figs 1 and 2). Neither patient deviated from the canonical sequence in exon 5. Amplified exon 4 of six normals was sequenced by the direct method, and the expected GGC codon for glycine 60 was found in all cases (Fig 1). The AGC mutant triplet was confirmed by using direct sequencing in the triplet of one of the patients. To our knowledge, this is the first report of direct sequencing of PCR products by the Sanger/Sequenase method by using unlabeled primers and processive incorporation of 35S-dATP. The gcAgc sequence generated by the mutation is the recognition site for the restriction enzyme BbvI. The generation of this new restriction site was confirmed for both patients (data not shown).

DISCUSSION

Two presumptively nonrelated hemophilia B patients demonstrating a defect in expression of the 2D5 epitope were chosen for this study from a set of 25 CRM+ samples. PCR-amplified exon 4 and exon 5 DNA from both patients was identical in size to that of corresponding exonic DNA amplified from normal individuals as evaluated by gel electrophoresis. This suggested that a single base change or very small deletion/insertion was responsible for the mutations.

DNA sequence analysis detected a single identical G-to-A transition at nucleotide 10,430 in exon 4 of the FIX gene in both patients as compared with previously published sequences. This mutation substitutes glycine 60 with serine in the patients' FIX (Fig 2). Although the PCR technique may lead to random artifactual mutations of amplified DNA sequences, the simultaneous occurrence of the same mutation in two independently amplified sequences is extremely unlikely. The detection of conserved sequences in amplified exon 4 of normal males rules out an unexpected "hot spot" for misincorporation by the Taq polymerase. The mutation observed probably is not a polymorphism because neither any published sequence for FIX nor the six normal exon 4 regions sequenced by by using PCR deviate from the accepted sequence in this region.

Fig 1. Nucleotide sequencing gels of a normal male (a) and patient 1 (b) for the area surrounding nucleotide 10,430 (A in 1b) in exon 4 of FIX. The normal male sequence (A) demonstrates results obtained by direct sequencing as described in Materials and Methods.

Fig 2. A schematic illustration of the first EGF-like domain of human FIX based on a tentative NMR-derived solution structure of human EGF. Conserved cysteines are solid circles; other conserved residues are indicated by shading. Glycine 60 (in the square), which begins a β-pleated sheet, is mutated to serine in FIX Durham. "β" indicates a β-hydroxyaspartic acid residue.
Table 1. Comparison of EGF-Like Domains in Several Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>Homology*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch</td>
<td>D</td>
<td>C</td>
<td>S</td>
<td>N P C - N G G - C - D - N S Y - C - C - P - G F - G K - C E - D 24</td>
<td></td>
</tr>
<tr>
<td>F.X</td>
<td>G</td>
<td>D</td>
<td>Q</td>
<td>C</td>
<td>E - P C - N - G - C K β - - - - Y - C - C - G F E G K N C E L 24</td>
</tr>
<tr>
<td>F.VII</td>
<td>D</td>
<td>Q</td>
<td>C</td>
<td>S</td>
<td>P C - N G G S C K β - - - - S Y - C - C - F E G - N C E 23</td>
</tr>
<tr>
<td>t-PA</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>C - N G G - C - - - - - S - C - C P - G F - G K - C E - D 17</td>
</tr>
<tr>
<td>F.XI:2</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>N P C L - G G - C - - - - - - - - - - - C - C P - G - G - C - D 15</td>
<td></td>
</tr>
<tr>
<td>F.XI:1</td>
<td>D</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>- P C - G G - C - - - - - - - - - - - C - C P - G - G - C - D 13</td>
</tr>
<tr>
<td>Human EGF</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>- C L - G G - C - - - - - - - Y - C - C - G - G - C - - - 12</td>
</tr>
<tr>
<td>Protein C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>S</td>
<td>- C - G - C - β - I - S - C - C - G - G - C - - - 13</td>
</tr>
<tr>
<td>Lin 12</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>S</td>
<td>P C - N - A - C - D - Y - C - C - G F E G - C E - 10</td>
</tr>
</tbody>
</table>

Protein sequences were extracted from the following sources: Notch XI, F.XI, F.VIIa, t-PA, EGF, F.XI:2, F.XI:1, Protein C, Lin 12. Insertions and deletions in sequences relative to human F.IX have not been indicated.

*Number of homologous residues identical to human F.IX.
†β indicates β-OH modification of residue.
The presence of a second mutation in an unsequenced region of both patients’ FIX gene cannot, in the strictest sense, be ruled out. The rare occurrence of mutations, as well as the comapping of the epitopic defect with the observed mutation, presents a strong argument against undetected additional mutations being responsible for hemophilia in these patients. For these reasons, we feel justified in assigning the name FIX Durham to this pair of identical mutant proteins and attributing their deficit in function to the observed mutation. Although the mutation responsible for FIX Durham is unique in its location, it is similar to FIX Chapel Hill, FIX Oxford/San Dimas, and several FVIII mutations in being the result of a single G-to-A transition occurring in a CG dinucleotide.4

Correlation of the altered primary sequence of FIX Durham with its abnormal FIX:C activity requires knowledge of both the spatial structure and function of EGF-like domains. FIX shares this domain with a variety of proteins including FXII, FX, FVII, proteins C and S, EGF, LDL receptor, and others including the products of developmental genes in invertebrates (notch and δ in Drosophila melanogaster, lin-12 in Caenorhabditis elegans).4.5 The only obvious functional relation between these diverse proteins is that they are either cell surface associated or act near or at a cell surface, possibly to anchor a circulating protein to a cell membrane.6 Whatever the function of the EGF-like domains in these proteins may be, the structural significance of glycine 60 in human FIX is emphasized by the conservation of this amino acid at a homologous position in both human- and insect-derived sequences. The only additional residue reported is alanine in some invertebrate proteins (Table 1). Based on an NMR-derived structure for human EGF, Gly 60 would be expected to mark the beginning of the first strand of a β-sheet structure characteristic of the amino terminal domain of EGF-like structures (Fig 2).12.23 Substitution of a polar serine for the glycine can be suspected to alter the secondary and/or tertiary structure of the region, perhaps by formation of an inappropriate hydrogen bond.

Currently the relationship of the mutation in FIX Durham to its loss of coagulant activity is unknown, but the interaction of the molecule with FVIII and endothelial cells as well as its degree of β-hydroxylation is of clear interest. The first EGF-like region of FIX (and closely related serine proteases of coagulation) contains a β-hydroxyaspartic acid residue at position 64 which may be associated with Ca2+ binding.24,25 FIX Alabama has a Gly-for-Asp 47 substitution at the amino terminal junction of exon 4. This protein has been reported to have an abnormal interaction with endothelial cells26 and FVIII (personal communication, H. Roberts). Abnormal interaction of FIX Durham with cellular receptors, Ca2+, and/or FVIII may explain the nature of its functional defect.

ACKNOWLEDGMENT

The authors are grateful to James E. Anderson for his expert technical assistance and Dr Edison Liu for helpful suggestions about direct sequencing of PCR products.

REFERENCES

18. Leytus SP, Foster DC, Kurachi K, Davie EW: Gene for
human factor X: A blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. Biochemistry 25:5098, 1986


20. Greenwald I: Lin-12, A nematode homeotic gene is homologous to a set of mammalian proteins that includes epidermal growth factor. Cell 43:583, 1985


Hemophilia B Durham: a mutation in the first EGF-like domain of factor IX that is characterized by polymerase chain reaction

PH Denton, DM Fowlkes, ST Lord and HM Reisner