A Codon 338 Nonsense Mutation in the Factor IX Gene in Unrelated Hemophilia B Patients: Factor IX<sub>338</sub> New York

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Hemophilia B is an X-linked recessive bleeding disorder resulting from a deficiency of the coagulation factor IX (FIX) protein activity, a vitamin K-dependent serine protease active in both the intrinsic and extrinsic coagulation systems. DNA analyses of the factor IX gene in two unrelated patients with severe hemophilia B, with a IX coagulant activity <1% and undetectable FIX antigen, detected the loss of the second TaqI site in exon h (VIII) in both individuals. Polymerase chain reaction (PCR) amplification of 576 base pairs of exon h (VIII) with cloning and dideoxy sequencing of cloned DNA from one hemophiliac revealed a single C → T transition in codon 338 that changes an arginine residue CGA to a nonsense codon TGA. Allele-specific oligonucleotide probe hybridization with a mutant (C → T) and a wild-type allele confirmed the same mutation in amplified genomic DNA of the second hemophilia patient. The C → T transition represents another example of mutation at a CpG dinucleotide. DNA polymorphism analysis of the FIX gene in both individuals revealed each to be on a separate FIX haplotype; therefore, predicting each to be a separate mutation event.© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Hematologic assays. FIX coagulant activity was measured on fresh citrated plasma samples by the one-stage assay and FIX antigen by Laurell quantitative immunoelectrophoresis. Polyclonal antibody to human FIX was obtained from CalBiochem-Behring, San Diego.

Restriction endonuclease analyses. High molecular weight DNA was isolated from peripheral blood leukocytes as previously described. Three micrograms of DNA were digested with the restriction endonucleases TaqI, XmnI, MspI, and DdeI, according to the manufacturer’s specifications (New England Biolabs, Beverly, MA), separated on 0.8% to 1.2% agarose gels, transferred to nitrocellulose filters, and hybridized to α<sup>-</sup>P-labeled DNA probes representing a 2.0 kb FIX cDNA, or a 5.3 kb random sequence probe 52A (DXS51), and autoradiographed as described.

DNA amplification. A 576-base pair (bp) segment of exon h (VIII) of the FIX gene was amplified by the polymerase chain reaction (PCR). Twenty-one base oligonucleotide primers representing a 5’ FIX sequence (5’GAACATATAATTGAGGAGACAC 3’) at 30,823 bp (exon h, codon 235) and a 3’ sequence (5’AATGATACCCTTGAATCT 3’) at 31,399 bp (3’ untranslated region) were synthesized on an Applied Biosystems 380B DNA synthesizer. One microgram of total genomic DNA was amplified with 80 picomoles of each primer and 2.5 l of Taq polymerase for

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30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) according to specifications of the manufacturer (Gene Amp, Perkin-Elmer Cetus). Each cycle consisted of a DNA denaturation at 94°C (one minute), annealing at 53°C (two minutes), and extension at 72°C (three minutes).

DNA sequence analysis. The 576-bp amplified DNA was purified by extraction from a low melting agarose gel by glass beads (Gene-Clean, Bio 101, La Jolla, CA). The DNA of propositus SII-1 was then cloned into the plasmid vector pGEM3zf (Promega Biotec, Madison, WI) by the following strategy. Purified amplified FIX DNA was phosphorylated with T4 polynucleotide kinase (New England Biolabs) and then phenol-chloroform extracted and ethanol precipitated. The vector was prepared by cleaving with the endonuclease HinclI and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, West Germany). Five hundred nanograms of blunt end insert was then ligated into 100 ng of the dephosphorylated vector by T4 DNA ligase for four hours.

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Restriction endonuclease analyses. DNA analyses for FIX restriction endonuclease polymorphic sites at DdeI, XmnI, TaqI, and MspI were analyzed with a FIX cDNA probe. Figure 1 illustrates that in family S, the propositus (III-1) has a FIX gene haplotype of (−− −−) at Ddel, XmnI, TaqI, MspI sites, respectively. His mother (II-2) and aunt (SII-1) are both homozygous at these sites. In family P, IV-1 and IV-2 have a FIX polymorphic haplotype of (−− + +). The analyses with TaqI also detected a unique 3.0 kb fragment in hemophiliacs SIII-1, PIV-1, and the carrier mother, SII-1 (Fig 2). PIV-2 also has the same 3.0 kb TaqI fragment (data not shown). A loss of the second TaqI site in FIX exon h (VIII) at codons 337-338 could generate the 3.0 kb fragment, as illustrated in the FIX gene map in Fig 2. Similar analyses of DNA from 30 hemophilia B patients (25 with FIX <0.01 U/mL and normal individuals (n = 35) did not detect the 3.0 kb fragment, therefore, suggest this was unlikely to be a polymorphic site.

DNA amplification and sequence analysis. FIX DNA sequences representing 576 bp of exon h (VIII) were amplified from 1 µg of total genomic DNA from SII-2, SIII-1, PIV-1, and a normal male control. Approximately 1 µg of the 576-bp fragment was generated representing over a million-fold amplification (data not shown). Subsequent cloning of the FIX sites noted above. The polymorphic FIX loci, 52A (TaqI) and FIX (Ddel, XmnI, TaqI, MspI) are noted as (+) for the presence of the restriction site and (−) for its absence. In family P, the propositi IV-1 and IV-2 have an FIXC of <1%. Haplotypes are formulated only at the FIX sites noted above. The same mutant 3.0 kb TaqI fragment was noted in subjects SII-2, SIII-1, PIV-1, and PIV-2.
FIX GENE NONSENSE MUTATION

![Gene Map](Image)

**Fig 2.** DNA analysis with TaqI and a FIX cDNA probe. The FIX gene map with TaqI cleavage sites and fragments detected by FIX cDNA is illustrated. Blot 2A shows DNA from the mother, SI-2 (lane 1), and son, SI-1 (lane 2). A unique 3.0-kb fragment is noted in both. The son lacks the normal 2.7-kb fragment. Loss of the second TaqI site (**) in exon h (VIII) would generate a 3.0-kb fragment as noted on the FIX gene map. Blot 2B, lane 1, again represents SI-2; lane 2 is PIV-1 who has lost the 2.7-kb TaqI fragment and gained a 3.0-kb fragment. Note that SI-2 has the TaqI (intron IV) 1.8-kb polymorphic fragment, while PIV-1 has the 1.3-kb fragment. Note the TaqI polymorphic site in intron IV.

the 576-bp insert from SIII-1 into the pGEM3zF plasmid vector yielded 10^3 recombinant white colonies per microgram of DNA. Dideoxy-DNA sequencing of the 576-bp cloned DNA from SIII-1 detected a single base change, a C->T transition, at codon 338 (Fig 3). This substitution changes an arginine codon (CGA) to a nonsense codon (TGA).

**Oligonucleotide probe hybridization.** In order to confirm that this base substitution was present in genomic DNA of SIII-1, SII-2, and for analysis of PIV-1, amplified DNA was hybridized to oligonucleotide probes representing the normal (wild-type) 21 base sequence and the C→T mutant sequence at codon 338. Figure 4 demonstrates that the carrier mother’s (SII-2) DNA hybridizes to both the normal and mutant C→T oligomers, the two hemophiliacs (SIII-1 and PIV-1) to only the C→T mutant oligomer, and a normal male control to the normal oligomer. No hybridization signal was noted with the G→A mutant oligomer in either patient (data not shown).

**Carrier diagnosis.** The half sister of SII-2 requested carrier diagnosis for hemophilia B. Because it was uncertain whether SI-2, the deceased maternal grandmother, was a carrier, conventional linkage analysis using FIX DNA polymorphisms was not possible. However, direct mutation analysis for the mutant 3.0-kb TaqI fragment was negative and her FIXC activity = 0.95 U/mL, thus confirming her noncarrier status (data not shown). Attempts to ascertain whether the mother, SII-2, represents a new mutation in this pedigree is limited by the fact that both maternal grandparents are deceased and analyses at four FIX intragenic DNA polymorphic loci were noninformative in both sisters. However, analyses at a linked random sequence polymorphic probe, S2A, which has a recombination fraction with the FIX gene of θ = 0.018, suggests both sisters may share a maternal X chromosome. This chromosome with the haplotype + - - - + in SII-1 (Fig 1) appears to be the background for the FIX 3.0-kb mutant fragment in SII-2. However, definitive identification of the origin of this mutation is not possible at this time.

**DISCUSSION**

In this study we have detected the same C→T base transition at codon 338 in the FIX genes of unrelated patients with severe hemophilia B. In addition, this mutation appears on a different FIX gene haplotype background in each patient, which suggests they represent two independent mutation events. We have used PCR amplification of specific FIX DNA sequences with cloning, sequencing, and allele-specific oligonucleotide probe hybridization to rapidly char-
One third of hemophiliacs represent sporadic mutations that arose spontaneously within recent generations and there is no apparent selection factor for maintaining hemophilia genes within a population; a large pool of different mutations can be expected. Clearly, mutations at CpG dinucleotide may be over represented and a strategy may be devised for rapid assessment of these sites in the FIX gene with PCR technology and allele-specific oligonucleotide probes, similar to the screening methods for β-thalassemia mutants within a specific population.43

The 576-bp exon h (VIII) amplification product contains seven CpG dinucleotides, three of which represent restriction endonuclease cleavage sites at codons 252 (TaqI), 338 (TaqI), and 403 (MspI). A rapid screening for potential mutants at these sites can thus be achieved with DNA amplification, restriction endonuclease analysis, and direct visualization in stained agarose or acrylamide gels. Confirmation of altered sites can then be done by genomic sequencing or ASO probe analysis.

Identification of the specific mutation producing hemophilia B within a pedigree clearly improves carrier and antenatal diagnosis. Although the use of FIX intragenic DNA polymorphisms allows carrier detection in 63% of Caucasian women, the method of linkage analysis requires an informative pedigree. In family S, linkage analysis using DNA polymorphisms for the requested carrier diagnosis is not possible because both maternal grandparents are deceased and neither sister is informative at intragenic FIX probes. However, coagulant assays of FIXC = 0.85 U/mL and FIXAg = 0.76 U/mL were inconclusive and she subsequently had a hemophilic son (V-1). Clearly mutation-specific analysis can now be rapidly achieved in both families by PCR technology with direct analysis for the codon 338

![DNA sequence analysis](attachment:image.png)
*Taq* I site by restriction endonuclease and/or ASO probe analysis.

A previous study by Poon et al also reported the use of *Taq* I variants for carrier detection in hemophilia B.\(^4\) The reported variant fragments of 1.8 kb and 3.0 kb represent loss of *Taq* I sites at codons 252 and 338, respectively, in exon h (VIII) in severe CRM-negative hemophilia B patients. Whether these variants also represent CpG mutants, as described herein and in references 20 and 25, can only be determined by sequence or ASO analyses.

Clearly, advances in molecular biology techniques, such as PCR, are accelerating the identification of mutants and in turn are also providing more rapid and accurate diagnostic tools in genetic diagnosis.

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