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An Insertion within the Factor IX Gene: Hemophilia $B_{El\ Salvador}$

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Summary

A patient with moderate to severe hemophilia B has been found to have a large insertion within his factor IX gene. The site of insertion is located in a DNA segment of ~0.8 kb between exon IV and an *Eco*RI site within intron D. The size of the DNA insertion is ~6 kb, and it contains at least two *Taq*I sites, two *Eco*RI sites, and one *Hin*dIII site. The insert probably originates from outside the FIX gene and does not represent an internal duplication. We propose that this abnormal FIX gene be called $FIX_{El\ Salvador}$ in recognition of the birthplace of the patient.

Introduction

Hemophilia B is an X-linked disease caused by a deficiency or dysfunction of factor IX, which participates in the middle phase of blood coagulation. The gene coding for factor IX has been cloned and sequenced (Choo et al. 1982; Kurachi and Davie 1982; Anson et al. 1984; Yoshitake 1985). Approximately one dozen discrete defects of the factor IX gene in patients with hemophilia B have been reported (Thompson 1986). These defects include complete (Gianelli et al. 1983) and partial gene deletions (Peake et al. 1984; Chen et al. 1985*b*) and point mutations (Noyes et al. 1983; Chen et al. 1985*a;* Rees et al. 1985; Bentley et al. 1986). Elucidation of these defects has provided important insights into the mechanisms that are responsible for hemophilia B.

This report documents another mechanism as a probable cause for hemophilia B, the insertion of a large (6-kb) segment of DNA into the gene coding for a deficient factor IX.

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Material and Methods

A 17-year-old male from El Salvador had ~1% normal factor IX clotting activity and was considered to be affected with moderate to severe hemophilia B. His factor IX antigen level, in his stored EDTA supernatant plasma, was ~6% of normal (0.06 unit/dl) according to a solid-phase immunoradiometric method performed by A. R. Thompson of the University of Washington. Other family members were not available for study because the subject is adopted.

Approximately 250 μg of DNA was isolated from 10 ml of blood anticoagulated with EDTA, and restriction-enzyme digestions of DNA, followed by agarose-gel electrophoresis and Southern transfer onto nitrocellulose filters, were performed as reported elsewhere (Chen et al. 1985*b*). The DNA fragments in the filter were hybridized with a factor IX DNA probe labeled with ^{32}P-deoxynucleotides, and the filter was washed and then exposed to Kodak AR film (Eastman Kodak Co., Rochester) between a pair of intensive screens at −70 for 5–10 days.

A cDNA probe and a genomic probe of factor IX were labeled with α-^{32}P-deoxynucleotides to 1–3 × 10^8 cpm/μg DNA in a nick-translation reaction (Kelly et al. 1970). The cDNA probe employed in this study has been reported elsewhere (Kurachi and Davie 1982) and includes the sequence coding for the entire signal peptide, the mature protein, and part of the 3′-noncoding sequence. The genomic DNA probe

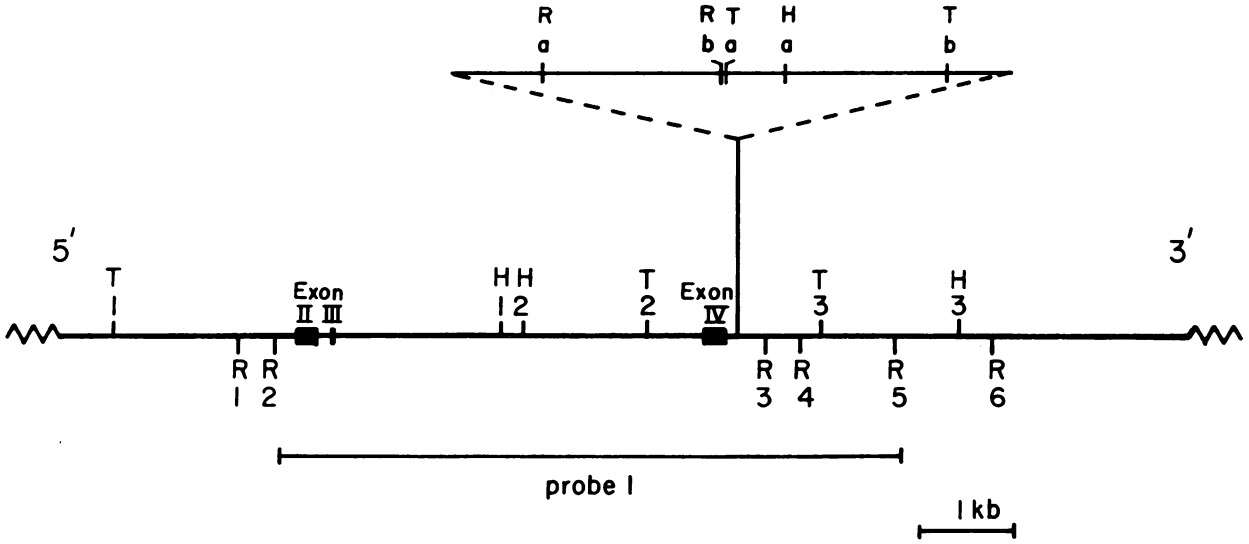

Figure 1 The site and length of insertion in the abnormal factor IX gene. The figure represents a portion of the normal organization diagramed in an earlier paper (Yoshitake et al. 1985). It demonstrates the *Eco*RI (R), *Taq*I (T), and *Hin*dIII (H) sites in the region. The length and position of the genomic probe are depicted as "probe 1." The inserted segment (~6 kb), including the proposed *Eco*RI, *Taq*I, and *Hin*dIII sites, is shown in the upper portion of the diagram.

(probe 1) was 6.2 kb in length and contained the sequence starting at the second *Eco*RI site (R-2 in fig. 1) through the fifth *Eco*RI site (R-5 in fig. 1). The probe contains exons II, III, and IV, the 3′ portion of intron A, introns B and C, and the beginning of intron D.

Results

When DNA samples from a normal male and the patient were evaluated by a Southern blot analysis after digestion with *Taq*I, *Eco*RI, and *Hin*dIII and probed with the cDNA, a difference in size between some of the restriction fragments was apparent (fig. 2). The 1.8-kb *Taq*I fragment present in normal DNA was replaced by a 3.9-kb fragment from the patient's DNA. Accordingly, the 4.8-kb fragment generated by *Eco*RI digestion was replaced by a strongly hybridizing 5.5-kb fragment, and the normal 4.3-kb fragment generated by *Hin*dIII was shifted to a 5.8-kb band in the patient's DNA. In the normal factor IX gene the 1.8-kb fragment, the 4.8-kb fragment, and the 4.3-kb fragment generated by cleavage with *Taq*I, *Eco*RI, and *Hin*dIII are all derived from the region containing exon IV of the factor IX gene.

When the genomic probe was hybridized with the same filter, the extent of the genomic reorganization in the patient's DNA was better visualized (fig. 2). The 1.8-kb band observed in cleavage of normal DNA with *Taq*I was replaced by newly generated 3.9-kb and 1.4-kb bands in patient DNA. The 1.4-kb fragment was not observed with the cDNA probe. The 4.8-kb band generated by the *Eco*RI digestion of normal DNA was replaced by a 5.5-kb band and a newly detected 3.6-kb band. The 4.3-kb fragment of the normal DNA produced by *Hin*dIII was now replaced with a 5.8-kb band and a new 4.6-kb fragment. These data indicate that an additional segment of DNA is inserted into the abnormal factor IX gene in the vicinity of exon IV.

Discussion

Previous abnormalities of the factor IX gene have been shown to be either deletions or base substitutions. In the present study, we present evidence that an alternative mechanism, a large insertion in the factor IX gene, is associated with the presence of hemophilia B.

The genomic DNA obtained from the patient contained a 5.3-kb fragment when analyzed with *Taq*I and a 1.0-kb fragment when analyzed with *Eco*RI (fig. 2). These fragments represent two segments of DNA, T_1-T_2 and R_4-R_5, within the factor IX gene (fig. 1). Both fragments were present in normal DNA and in the patient. The insertion, therefore, must lie in the region of DNA between T_2 and R_4. If the insertion site was between T_2 and exon IV, both the

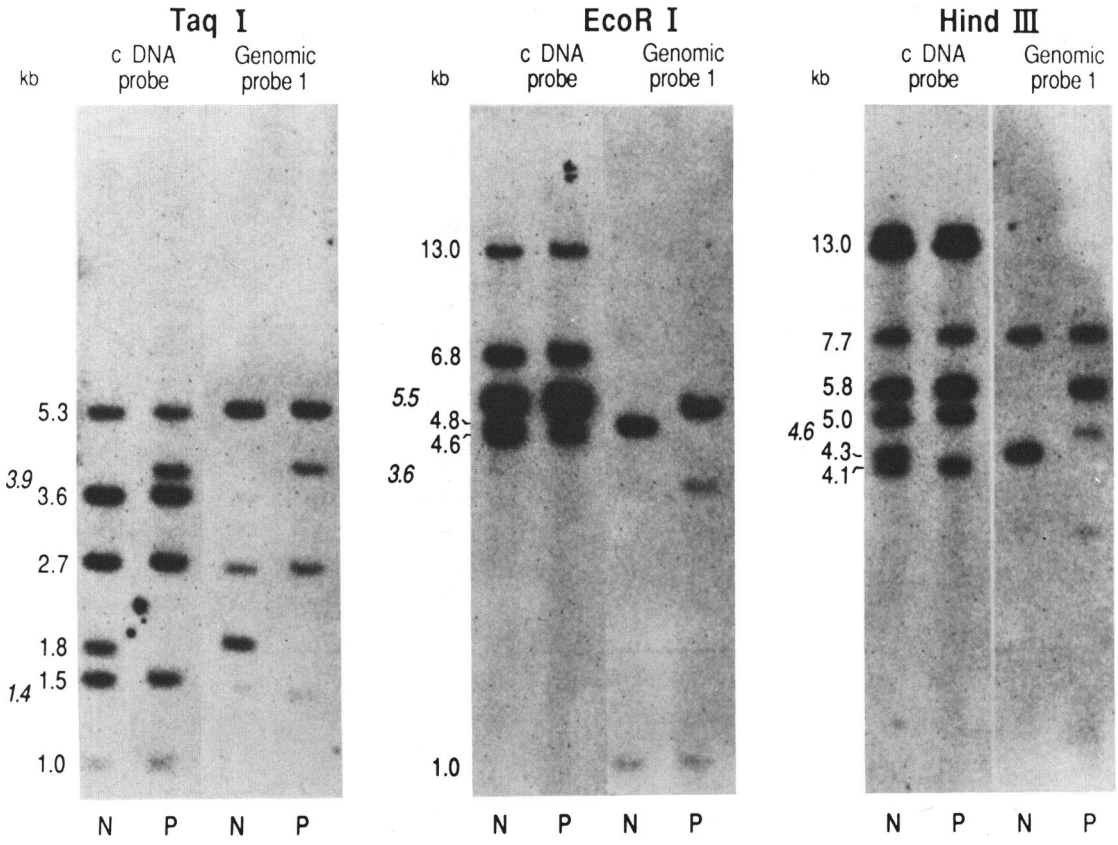

Figure 2 Southern blot analysis of DNA, from a normal person (N) and from the patient (P), digested with *Taq*I, *Eco*RI, and *Hin*dIII and hybridized with the cDNA probe and with the genomic probe 1.

3.6-kb and 5.5-kb fragments of the patient's DNA that were generated by *Eco*RI digestion would have hybridized with the cDNA probe. The fact that a 3.6-kb fragment did not hybridize with the cDNA probe indicates that the 3.6-kb fragment was from a portion of the intron and that the insertion site was not located between T_2 and exon IV. Alternatively, if the insertion site was between exon IV and R_4 in DNA from the patient, the genomic probe should hybridize with the 5.5-kb and 3.6-kb segments and the cDNA probe should only hybridize with the 5.5-kb fragment. Our data are consistent with the latter. The distance between exon IV and R_4 is ~0.8 kb.

Southern blot analyses of the patient's genomic DNA obtained after digestion with *Hin*dIII showed that the normal 4.3-kb fragment was replaced by two fragments with sizes of 5.8 and 4.6 kb (fig. 2). The difference between the sum of these fragments in the patient (~10.4 kb) and the normal size (4.3 kb) is 6.1 kb. This represents the minimum estimate of the size of the insertion. Analyses of restriction fragments generated with *Taq*I and *Eco*RI, however, only detect ~4 kb of additional DNA within the patient's factor IX gene. This most likely is due to the existence of two or more *Taq*I and *Eco*RI sites within the inserted segment of DNA. This is hypothetically shown in the insertional map within figure 1. Digestions with two other enzymes, *Xba*I and *Spt*I, also demonstrate the existence of a DNA segment of ~6 kb into the factor IX gene of the patient (data not shown). Further precision regarding the size and site of this insertion near exon IV will have to be determined by sequencing the inserted DNA fragment and its junction regions.

It is possible that the insertion could represent a rare variant; however, its size (6.0 kb) and the insertion site at or near the 3′ end of exon IV strongly suggest that the abnormality is associated with the hemophilia. No similar variation has been found either in examination of more than 300 normal X chromosomes in this laboratory or by others evaluating the FIX gene.

Exon IV codes for amino acids 47–85 of the nor-

mal factor IX protein (Kurachi and Davie 1982). This amino acid sequence forms the first of two "growth factor–like domains." An insertion near the splice junction between intron D and exon IV might alter the splicing of this region and result in an abnormal factor IX. Such splicing alterations have been shown to exist for β-thalassemia (Orkin 1983) and in another hemophilia B patient (Rees et al. 1985). In both cases, the defects were the result of a nucleotide substitution rather than a consequence of insertion.

Expansion of gene size has usually occurred as a result of unequal crossing-over between homologous sister chromatids and has been shown to result in such events as gene duplication or major gene rearrangements. Examples include the classic model of α-thalassemia or the recent documentation of the evolution of red-green color blindness genes (Nathans et al. 1986). In these examples the gene expansion involves contiguous DNA segments. Small duplications (10–100 bp) may occur within introns and not interfere with gene function. They are usually only recognized as a polymorphism for a specific endonuclease within a population (Yoshitake et al. 1985). Large insertions within a human gene that results in malfunction have been documented infrequently. Yang et al. (1987) have reported, in a male with HGPRT deficiency, an internal duplication of exons 2 and 3 of the gene coding for HGPRT. The insertion reported here appears to be from outside the FIX gene, since it does not contain a FIX exon and since the restriction-enzyme sites are not similar to those that exist in other parts of the gene.

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