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Factor $IX_{Madrid\ 2}$: A Deletion/Insertion in Factor IX Gene Which Abolishes the Sequence of the Donor Junction at the Exon IV–Intron d Splice Site

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Summary

DNA from a patient with severe hemophilia B was evaluated by RFLP analysis, producing results which suggested the existence of a partial deletion within the factor IX gene. The deletion was further localized and characterized by PCR amplification and sequencing. The altered allele has a 4,442-bp deletion which removes both the donor splice site located at the 5′ end of intron d and the two last coding nucleotides located at the 3′ end of exon IV in the normal factor IX gene; this fragment has been replaced by a 47-bp sequence from the normal factor IX gene, although this fragment has been inserted in inverted orientation. Two homologous sequences have been discovered at the ends of the deleted DNA fragment.

Introduction

Hemophilia B is an X-linked bleeding disorder caused by either a deficiency or an altered production of factor IX, which is the precursor of a serine protease that participates in the middle phase of blood coagulation. Hemophilia B is a heterogeneous disease, with a large number of discrete defects described for the factor IX gene. These defects include complete (Anson et al. 1988; Ludwig et al. 1989) and partial gene deletions (Giannelli et al. 1983; Chen et al. 1985), DNA segment insertion into the factor IX gene (Chen et al. 1988), as well as point mutations or short deletions altering different positions within the multiple domains of the factor IX primary structure (Giannelli et al. 1991).

The present report documents a new mutation as a probable cause for hemophilia B. The mutation consists of a deletion/insertion in the factor IX gene. This deletion/insertion eliminates the sequence of the exon IV–intron d splice site and blocks the maturation of factor IX precursor mRNA. A possible mechanism for the origin of this molecular defect is discussed.

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Material and Methods

A 17-year-old male affected with severe hemophilia B had 0% of normal factor IX:Ac; his factor IX:Ag level was <1% of normal; and he did not develop inhibitor antibodies to factor IX. Other family members available for study were the patient's sister and parents. There was no family history of the disorder. Peripheral blood samples were obtained from the four members of the family B07, as well as from normal control subjects. Approximately 200 μg of DNA was isolated from 10 ml of blood anticoagulated with EDTA. Restriction-enzyme digestion, blotting, radioactive labeling of the probes by the random priming method, hybridization, and autoradiography were performed according to standard procedures (Maniatis et al. 1982). Probes VIII, XIII, and I consisted in three factor IX gene fragments, provided by Dr. G. G. Brownlee of Oxford University. The amplification of the altered DNA region was performed on 0.1 μg of genomic DNA by using PCR, according to a method described elsewhere (Saiki et al. 1988). The conditions for amplification were 1 min at 93°C for denatur-

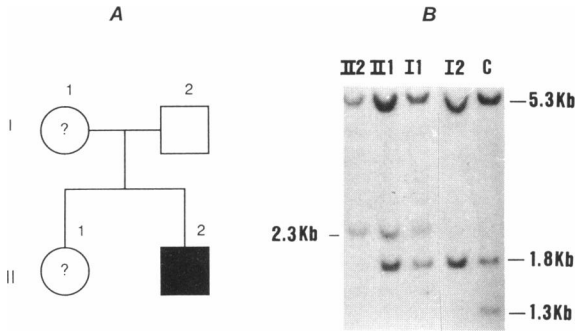

Figure 1 *A*, Pedigree of factor IX–deficient family B07. *B*, Southern blot analysis of DNAs digested with *TaqI* and hybridized to factor IX intragenic probe VIII. Lane C, Normal polymorphic fragments of 1.8 kb and 1.3 kb detected in DNA control. Lanes II2, II1, and I1, Abnormal fragment of 2.3 kb. Each lane contained approximately 5 mg of DNA sample.

ation, 30 s at 62°C for annealing, and 1.5 min at 72°C for extension. The two amplification sequence primers (23 mer) used in the PCR reaction were primer A—5′ GGCATTCTAAGCAGTTTACGTGC 3′ (nucleotides 10341–10363 in the factor IX gene)—and primer B—5′ AATGGAGCTGGAGGCCTATCATCC 3′ (nucleotides 15539–15516 in factor IX gene) Yoshitake et al. (1985). PCR products were subcloned into pUC18 plasmid, and both strands from two independent clones were sequenced by the dideoxy chain-termination method (Sanger et al. 1977).

Results

When DNA samples from four members of family B07, whose pedigree is depicted in figure 1*A*, were evaluated by Southern blot analysis, several abnormal restriction fragments were found. Figure 1*B* shows one of these band-position changes identified, which was observed by using the combination *TaqI*/probe VIII. It consisted in an unusual 2.3-kb restriction fragment which was found in the patient's DNA (fig. 1*B*, lane II2) as well as in the patient's mother's DNA (fig. 1*B*, lane II) and in the patient's sister's DNA (fig. 1*B*, lane III). Determination of the length of the abnormal fragments and comparison with the factor IX restriction map previously published (Yoshitake et al. 1985) allowed us both to estimate the extent of the deletion as approximately 4.5 kb and to define its location in a position nearby the exon IV–intron d junction of the factor IX gene (see diagram presented in fig. 2).

PCR amplification performed on the patient's DNA as described in Material and Method produced an amplified fragment of approximately 750 bp, while the two primers anneal to locations which are 5,198 bp apart within the normal gene sequence. Alignment of the amplified fragment's nucleotide sequence with the sequence of the normal factor IX gene, which is shown in figure 3*A*, revealed that the extension of the deletion was precisely 4,442 bp; it removes both a portion of intron d and the last two coding nucleotides at the 3′ end of exon IV. This mutation therefore deleted the consensus splicing sequence for precursor mRNA at the exon IV–intron d junction. This sequence feature predicts an abnormally processed factor IX mRNA as a product of the deletion. We have also found a 47-bp sequence, corresponding to an intron d portion lost in the deletion (positions 10523–10569 in the normal factor IX gene), which has been inserted in inverted

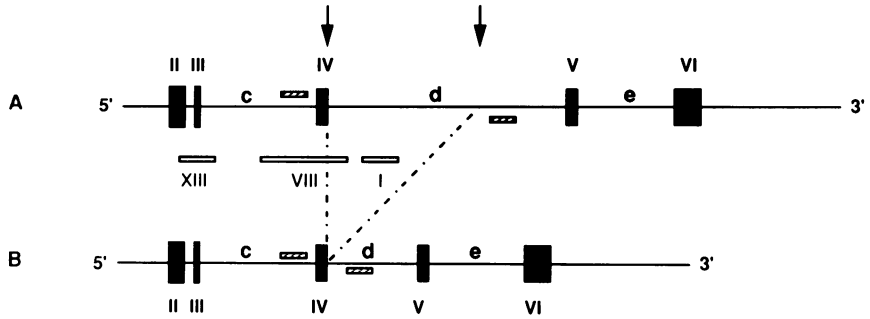

Figure 2 Localization of intragenic deletion in factor $IX_{Madrid\ 2}$ mutation. *A*, Portion of normal factor IX gene map, in which exons are shown as solid regions labeled with roman numerals (II–VI) and in which two arrows indicate approximate position of deletion breakpoints. Below, the sites of the genomic probes used (XIII, VIII, and I) are depicted as unblackened horizontal bars. *B*, Equivalent factor IX gene region with partial deletion delineated in patient. The sites of the oligonucleotides used in the PCR amplification are shown as blackened horizontal bars at the boundaries of the deletion.

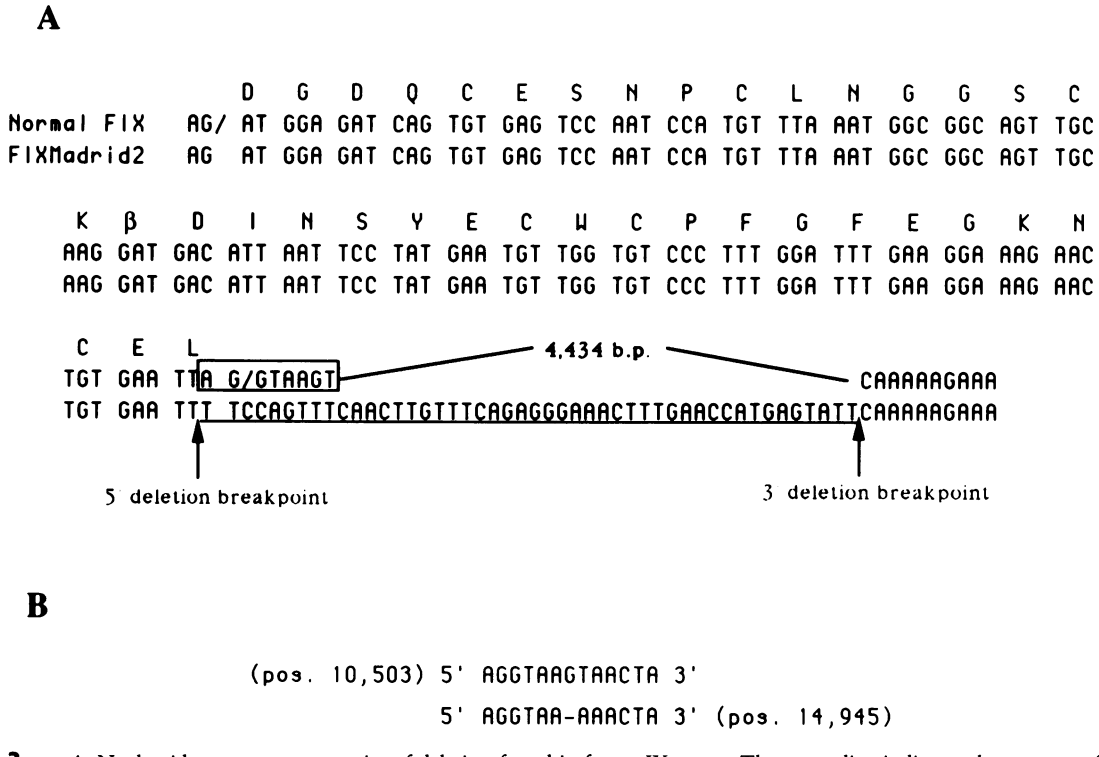

Figure 3 *A*, Nucleotide sequence across site of deletion found in factor $IX_{Madrid\ 2}$. The upper line indicates the sequence from the normal factor IX, according to Yoshitake et al. (1985), and includes also the amino acid translation of exon IV sequence. The bottom line corresponds to the sequence of the altered allele. The deletion suppressed the nucleotide sequence between positions 10503 and 14945 within the factor IX gene (Yoshitake et al. 1985). The inserted 47-bp sequence (underlined) is identical to the intron d sequence between positions 10526 and 10570 in the normal factor IX gene but is in inverted orientation. *B*, Diagram showing 13- and 12-bp nucleotide sequences located at the 5′ and 3′ ends of the deleted fragment and alignment of both nucleotide sequences, revealing 11 identical nucleotides.

orientation at the deletion breakpoint. Since such a mutation had not been previously reported, we named it factor $IX_{Madrid\ 2}$. Analysis of the nucleotide sequence of the fragment removed by the mutation revealed also the existence of two homologous sequences of 13 and 12 bp, located, respectively, at the 5′ and 3′ ends of the deleted DNA fragment found as direct repeats (see the sequence alignment shown in fig. 3*B*).

Discussion

The alteration in the factor IX gene we describe is undoubtedly the cause of hemophilia B, since it removes four nucleotides (AGGT) from the sequence of the splice donor junction 3′ of exon IV in the normal gene. Therefore, we have diagnosed both the patient's mother and the patient's sister as obligate carriers of the disease. Previously, several hemophilia B patients with a splice-site alteration have been reported. In some of those cases, the mutation consisted of single-nucleotide changes both within the obligatory GT of the donor splice site at the 3′ site of either exon VI (Rees et al. 1985) or exon III (Brownlee 1986) and within the obligatory AG of the acceptor splice site at the 5′ site of exon VIII (Matsushita et al. 1989). All these mutations were associated with severe hemophilia b, indicating the relevance of an invariant sequence at the exon-intron splice site. Tetranucleotide deletions in the 3′ acceptor splice consensus of intron d (Koeberl et al. 1989) and within the 5′ donor splice consensus of intron b near exon II (Poort et al. 1990) have been also found.

A number of factor IX gene deletions have been reported previously as the cause of hemophilia B, but in very few cases have the boundaries of the deleted sequence been characterized. Previously reported mutations include factor $IX_{London\ I}$ (Green et al. 1988), for which a nonhomologous, random recombination was claimed as the mechanism for the 23-kb deletion, and factor $IX_{Seattle\ I}$ (Chen and Scott 1990), with a 10-kb intragenic deletion. In the latter, a 13-bp sequence at the deletion junction is homologous to two 14-bp

identical sequences localized 10 kb apart in introns d and f of the normal factor IX gene. The authors propose that genetic recombination has occurred between the two homologous sequences, resulting in the gene deletion. It is interesting that we have discovered at the ends of the deleted region in factor $IX_{Madrid\ 2}$ two homologous sequences of 13 and 12 nucleotides, sequences which are present in direct repeats in the normal factor IX gene (fig. 3*B*). We postulate that a recombinational event based on the nucleotide pairing at the homologous sequences could be responsible in part for the observed molecular defect. Nevertheless factor $IX_{Madrid\ 2}$ contains also a 47-bp insertion at the deletion breakpoint, and this fact excludes the participation of a single-step mutational mechanism. Obviously, we cannot rule out other alternative mechanisms, such as slipped mispairing during replication (Efstradiatis et al. 1980).

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References

Anson D, Blake D, Winship P, Dirnbaum D, Brownlee GG (1988) Nullisomic deletion of the mcf.2 transforming gene in two haemophilia B patients. EMBO J 7:2795–2799

Brownlee GG (1986) The molecular genetics of hemophilia A and B. J Cell Sci 4 [Suppl]: 445–458

Chen S-H, Scott CR (1990) Recombination between two 14-bp homologous sequences as the mechanism for gene deletion in factor $IX_{Seattle\ 1}$. Am J Hum Genet 47:1020–1022

Chen S-H, Scott CR, Edson JR, Kurachi K (1988) An insertion within the factor IX gene: hemophilia $B_{El\ Salvador}$. Am J Hum Genet 42:581–584

Chen SH, Yoshitake S, Chance PH, Bray G, Thompson A, Scott C, Kurachi K (1985) An intragenic deletion of the factor IX gene in a family with hemophilia B. J Clin Invest 76:2161–2164

Efstradiatis A, Posakony JW, Maniatis T, Lawn RM, O'Connell C, Spritz RA, De Riel JK, et al (1980) The structure and the evolution of human beta globin gene family. Cell 21:653–668

Giannelli F, Choo KH, Rees D, Boyd Y, Rizza C, Brownlee G (1983) Gene deletions in patients with haemophilia B and anti-factor IX antibodies. Nature 303:181–182

Giannelli F, Green PM, High KA, Sommer S, Lillicrap DP, Ludwig M, Olek K, et al (1991) Haemophilia B: database of point mutations and short additions and deletions—second edition. Nucleic Acids Res 19:2193–2219

Green PM, Bentley DR, Mibashan RS, Giannelli F (1988) Partial deletion by illegitimate recombination of the factor IX gene in a haemophilia B family with two inhibitor patients. Mol Biol Med 5:95–106

Koeberl DD, Bottema CDK, Buerstedde J-M, Sommer SS (1989) Functionally important regions of the factor IX gene have a low rate of polymorphism and a high rate of mutation in the dinucleotide CpG. Am J Hum Genet 45: 448–457

Ludwig M, Schwaab R, Eigel A, Horst J, Egli H, Brackman H-H, Olek K (1989) Identification of a single nucleotide C-to-T transition and five different deletions in patients with severe hemophilia B. Am J Hum Genet 45:115–122

Maniatis AT, Fritsch ET, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Matsushita T, Tanimoto M, Yamamoto K, Sugiura I, Hamaguchi M, Takamatsu J, Saito N (1989) Nucleotide sequence analysis of hemophilia B with the inhibitor phenotype. Blood 74 [Suppl 1]: 251a

Poort SR, Briet E, Bertina RM, Reitsma PH (1990) Two mutations of the factor IX gene including a donor splice consensus deletion and a point mutation in a Dutch patient with severe hemophilia B. Thromb Haemost 64: 379–384

Rees D, Rizza C, Brownlee G (1985) Haemophilia B caused by a point mutation in a donor splice junction of the human factor IX gene. Nature 316:643–645

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mulli KB, et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sanger F, Nicklen S, Coulson AR (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560–564

Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K (1985) Nucleotide sequence of the gene for human factor IX (anti hemophilic factor B). Biochemistry 24:3736–3750