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Maternal Duplication Associated with Gene Deletion in Sporadic Hemophilia

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Summary

Sporadic occurrences of X-linked disorders can give insights into mutagenesis in man. In a case of sporadic hemophilia, associated with a partial deletion of the factor VIII gene, an unexpected inheritance pattern of gene rearrangements was observed. The factor VIII gene was found to be partially duplicated in the hemophiliac's mother. A pedigree analysis indicates that the mother has contributed both aberrant genes as well as the normal gene to her offspring. One simple model for the evolution of the deletion in this family is that the duplication is the precursor to the deletion.

Introduction

Hemophilia A is an X-linked bleeding disorder that has long been recognized as an excellent model for mutagenesis in man (Haldane 1935). The disease is caused by a defect in blood coagulation factor VIII and shows a wide range in clinical severity, resulting from heterogeneity in factor VIII gene mutations. (Antonarakis et al. 1985; Gitschier et al. 1985*b*, 1986; Youssoufian et al. 1987). Up to one-third of hemophilia cases occur "sporadically" in families with no prior history of the disease, reflecting the introduction of new mutations into the population (Haldane 1935). Investigation of these sporadic cases may help to answer questions about mutagenesis in man, such as whether mutations occur more commonly in male or female gametes, whether there is an effect of age on mutations, or whether either of these parameters has an effect on the type of mutation generated. These studies may also uncover unexpected features of mutagenesis.

During the pedigree analysis of one sporadic case of hemophilia, an unusual pattern of inheritance of factor VIII gene rearrangements was observed. The hemophilia in the patient is associated with a partial factor VIII gene deletion. Unexpectedly, a partially duplicated factor VIII gene was discovered in the mother. The mother has contributed normal, duplicated, and deleted chromosomes to her offspring. A simple model based on extended pedigree data is that the duplication is the precursor to the deletion.

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Material and Methods

Blood was obtained from individuals by venipuncture following informed consent. DNA was extracted from peripheral blood leukocytes as described by Bell et al. (1981). DNA was also extracted from a control human cell line GM1202A (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository). For Southern blot analysis, 5 μg genomic DNA was digested with restriction enzymes *Bam*HI, *Sst*I, *Taq*I, *Kpn*I, or *Eco*RI and separated by electrophoresis on 0.5% agarose gels. DNA was transferred to nitrocellulose (Southern 1975) and hybridized in 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate), 50% formamide, 1 × Denhardt's (100 × Denhardt's = 20% albumin, 20% polyvinylpyrrolidone, 20% ficoll), 1 g boiled, sonicated salmon sperm DNA/liter, 50 mM sodium phosphate, pH 6.8, and 10% dextran sulfate with ^{32}P-labeled DNA probe at approximately 500,000 cpm/ml hybridization solution. For Southern blot analysis and for library screening, a variety of single-copy probe fragments were derived from factor VIII genomic clones (Gitschier et al. 1984) including the following: a 1.2-kb *Sau*3AI fragment of p114.1; a 1.1-kb *Bam*HI/*Eco*RI fragment of p482.1; a 1.6-kb *Bam*HI

fragment of p482.4; 1.1-kb *Eco*RI/*Sst*I and 9.6-kb *Eco*RI fragments of p482.6; a 1.1-kb *Bam*HI/*Eco*RI fragment of p542.6; and a 2.7-kb *Eco*RI fragment of p542.8. One probe was derived from a portion of the factor VIII cDNA containing exons 21–26, a 783-bp *Apa*I/*Eco*RI fragment (Wood et al. 1984). The St14 probe is a 3.0-kb *Eco*RI fragment of the St14 clone reported by Oberle et al. (1985).

By detailed Southern blot analysis of the H96 and maternal DNA samples, predictions could be made about the sizes of fragments containing the deletion and duplication boundaries. The deletion junction in H96 DNA is contained on a 1.0-kb *Eco*RI fragment, the 5′ duplication junction in maternal DNA is contained on a 10.5-kb *Bam*HI fragment, and the 3′ maternal duplication junction is contained on a 9.0-kb *Bam*HI fragment. Thus, these fragments were isolated and appropriate regions were sequenced by the following strategies: The 1.0-kb deletion junction fragment from hemophiliac H96 was isolated by cloning *Eco*RI-digested, size-fractionated H96 DNA into lambda gt10 arms (Stratagene). The resulting library of 500,000 recombinant phage was screened with the 1.1-kb *Bam*HI/*Eco*RI probe fragment of p542.6. Nine positives contained the expected junction fragment. The 10.5-kb *Bam*HI fragment corresponding to the 5′ duplication junction was isolated by cloning unfractionated *Bam*HI-digested maternal DNA into lambda FIX vector (Stratagene). The resulting library of 1.6 million clones was screened with the 1.6-kb *Bam*HI probe fragment of p482.4. To ensure that the fragment containing the boundary was obtained, the eight positive clones were rescreened with a probe that should also be contained on the junction fragment, a 2.7-kb *Eco*RI probe fragment of p542.8. Three 5′ junction clones resulted. The 9.0-kb *Bam*HI fragment corresponding to the 3′ duplication junction was isolated by cloning size-fractionated, *Bam*HI-digested maternal DNA into lambda EMBL3 vector arms (Stratagene) and screening the library with the 783-bp *Apa*I/*Eco*RI cDNA fragment. Five positives were recovered from screening a library of 100,000 recombinant phage. The deletion and duplication breakpoints were mapped in more detail in the cloned fragments. Small regions containing the breakpoints, as well as the normal DNA corresponding to these regions, were cloned into M13 vectors and sequenced by the dideoxy-chain termination method of Sanger et al. (1977).

Results and Discussion

Deletion of exons 23–25 was previously observed in the factor VIII gene of a patient with sporadic hemophilia A (patient H96; Gitschier et al. 1985*b*). Figure 1 shows a Southern blot of *Bam*HI-digested genomic DNAs of the H96 family members, hybridized to a factor VIII cDNA fragment including exons 21–26 (Gitschier et al. 1984; Wood et al. 1984). The three hybridizing bands correspond to exons 21–22, 23–25, and 26 in the normal gene. Exons 23–25 are absent in the hemophilia DNA sample (III-1), and the exon 21–22 band is 12 kb, rather than the normal 9.9 kb in length, serving as a marker for the deletion in the pedigree analysis. Two nonhemophilic brothers (III-6 and III-7) show the expected, normal pattern, as do two sisters (III-3 and III-5) and the maternal siblings (II-3, II-4, and II-5). Sister III-4 is heterozygous for the deletion, which was determined to be indistinguishable from that in the patient by Southern blots with a series of enzymes and probes. The fact that two siblings have inherited the

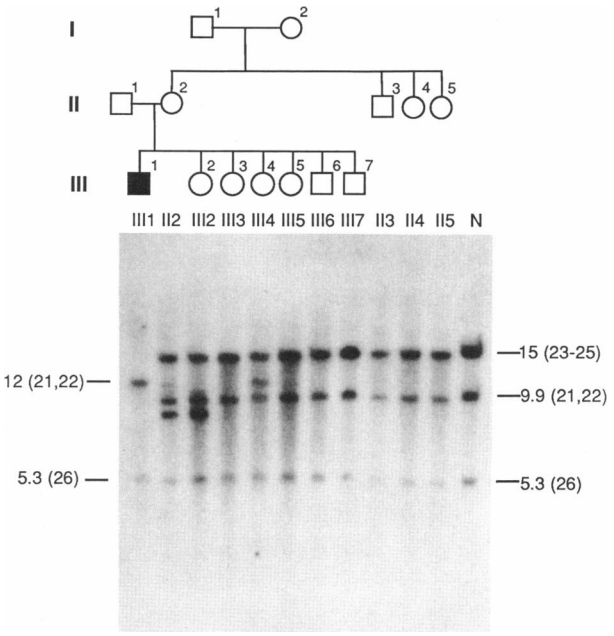

Figure 1 Southern blot analysis of factor VIII genes in the H96 family. A control DNA sample from an individual with a normal factor VIII gene is shown in lane N. Genomic DNA was digested with *Bam*HI, and the blot was hybridized to a factor VIII, 783-bp *Apa*I/*Eco*RI, cDNA probe containing exons 21–26. Sizes of the hybridizing fragments for the deleted and normal genes are given, with the corresponding exons noted in parentheses. Exons 23–25 are deleted in patient III-1, and exons 21 and 22 are contained on 9.9- and 12-kb fragments in the normal and deleted genes, respectively. The hybridization pattern in II-2 and III-2 is indicative of a different type of factor VIII gene rearrangement in the maternal and sister's chromosomes.

deletion demonstrates that the deletion event was not an isolated meiotic occurrence in the maternal gametes.

Unexpectedly, the genomic DNA samples of the mother (II-2) and one sister (III-2) show a pattern of hybridization that is different from both the normal and deletion patterns. As the experiments presented below demonstrate, the aberrant bands in these samples reflect another factor VIII gene rearrangement for which the mother and sister are heterozygous. The mother has contributed all three types of genes to her offspring, including the deleted gene to two individuals, indicating her germ line is mosaic for the normal, deleted, and rearranged genes. Indeed, her somatic tissue may be mosaic as well, since the 12-kb deletion band is present faintly in her blood sample but does not appear in that of her daughter (III-2; see fig. 1).

A comparison of the mother's DNA and that of the normal control digested with other restriction enzymes confirmed a gene rearrangement, as shown in figure 2. In the normal factor VIII gene, exons 23 and 24 are contained on a *Taq*I fragment of 1.4 kb and on a *Bam*HI fragment of 15 kb. (Digestion with *Sst*I gave comparable results; data not shown.) The mother appears to be heterozygous for the 1.4-kb *Taq*I fragment and for a pair of novel *Taq*I fragments (sizes 4.2 and 1.2 kb), implicating an insertion of DNA sequences between exons 23 and 24. Similarly, 9.0- and 10.5-kb *Bam*HI fragments replace the normal 15-kb fragment in one of her chromosomes. To test the insertion hypothesis and to determine the origin of the DNA inserted between the exons, the aberrant 9.0- and 10.5-kb *Bam*HI fragments were isolated by cloning into lambda vectors. The 9.0-kb *Bam*HI fragment was found to contain exons 24 and 25 adjacent to DNA sequences of unknown origin. A single-copy fragment was isolated from the 5′ adjacent DNA (indicated by hatched lines

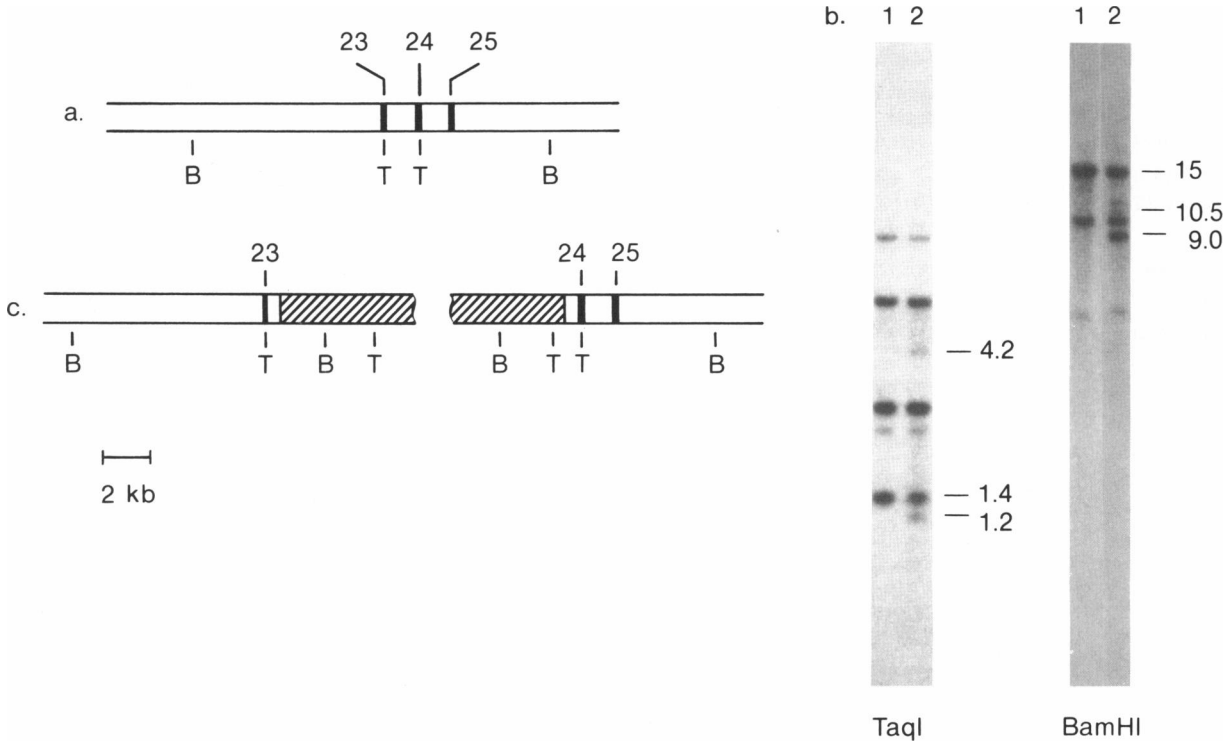

Figure 2 Comparison of normal and maternal VIII genes. In the normal gene (*a*), a 1.4-kb *Taq*I fragment contains exons 23 and 24, and a 15-kb *Bam*HI fragment contains exons 23–25 (T represents *Taq*I sites and B represents *Bam*HI sites). Southern blots (*b*) of *Taq*I- and *Bam*HI-digested DNAs from a control cell line (lane 1) and from the patient's mother (lane 2) were hybridized to an exon 21–26 cDNA probe fragment as in fig. 1. The mother is heterozygous for a gene rearrangement involving the 1.4-kb *Taq*I and 15-kb *Bam*HI fragments, resulting in pairs of novel bands. In the aberrant maternal gene (*c*), exons 23 and 24 are located on 4.2- and 1.2-kb *Taq*I fragments, respectively, and exons 23 and 24–25 are divided onto separate *Bam*HI fragments of 10.5 and 9.0 kb, respectively. The hatched region indicates the DNA inserted between exons 23 and 24 in the mother's gene. The 9.0- and 10.5-kb *Bam*HI fragments were isolated by cloning to analyze the maternal rearrangement in detail. Clones were authenticated by mapping and sequencing compared to normal factor VIII DNA.

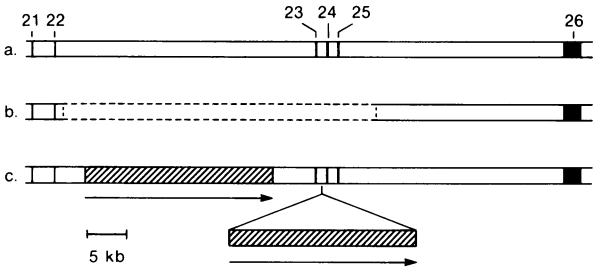

Figure 3 Structural comparison of normal factor VIII gene with deleted and duplicated genes in H96 family. Normally the coding region of the factor VIII gene is divided among 26 exons spanning 186 kb, the exon 21–26–containing portion of which is depicted (*a*). The gene in patient H96 (*b*) is deleted for 39 kb including exons 23–25, as indicated by the dashed line. The mother's gene (*c*) is duplicated for 23 kb of intron 22 DNA; the hatched area indicates the region of intron 22 DNA which has been "copied" and "inserted" between exons 23 and 24. The arrows indicate that the duplication causes a direct repeat of 23 kb, separated by a 6-kb spacer containing exon 23. The extent of the deletion is greater than the entire duplicated region in the maternal gene by 3 kb and 7 kb in the 5′ and 3′ directions, respectively.

in fig. 2*c*) and was used to probe a panel of all factor VIII genomic clones and male and female genomic DNAs. Under stringent conditions, it hybridized to a factor VIII sequence that is normally located in intron 22, between exons 22 and 23. Extensive Southern blot mapping using a variety of single-copy, intron 22 probes indicated that 23 kb of intron 22 sequence is duplicated and is inserted between exons 23 and 24. Figure 3 shows the resulting gene structure, a 23-kb direct repeat, separated by a 6-kb spacer. Mapping, cross-hybridization, and sequence analysis of the cloned 10.5-kb *Bam*HI fragment confirmed the duplicated structure.

As shown in figure 3, both copies of the duplication as well as 10 kb of neighboring sequences are lost in the deletion, suggesting that the duplication and deletion are probably not reciprocal recombination events. The tightly linked and highly polymorphic DNA marker St14 (DXS52; Oberle et al. 1985) was used to help clarify the relationship between the two genes. (The family was found to be uninformative at the intragenic *Bcl*I site [Gitschier et al. 1985*a*], and the *Xba*I site [Wion et al. 1986] is lost with the deletion.) Figure 4 demonstrates that the St14 allele 2 segregates with both deleted and duplicated genes in the nuclear family and allele 6 with the normal gene in every case except daughter III-5. The data suggest that the duplicated and deleted genes were produced by events occurring on the same X chromosome. The exceptional case is likely due to a recombination between St14 and the factor VIII gene, as these loci are known to be approximately 4 cM apart (Peake and Bloom 1986). Allele 2 is associated with a normal factor VIII gene in the maternal siblings, consistent with an appearance of a new mutation in the mother's DNA. The analysis also indicates that the aberrant genes are associated with the chromosome inherited from the maternal grandfather.

Although other explanations are possible, a simple working model can be proposed to account for the peculiar inheritance of factor VIII gene rearrangements in the H96 family. In this model, the duplication oc-

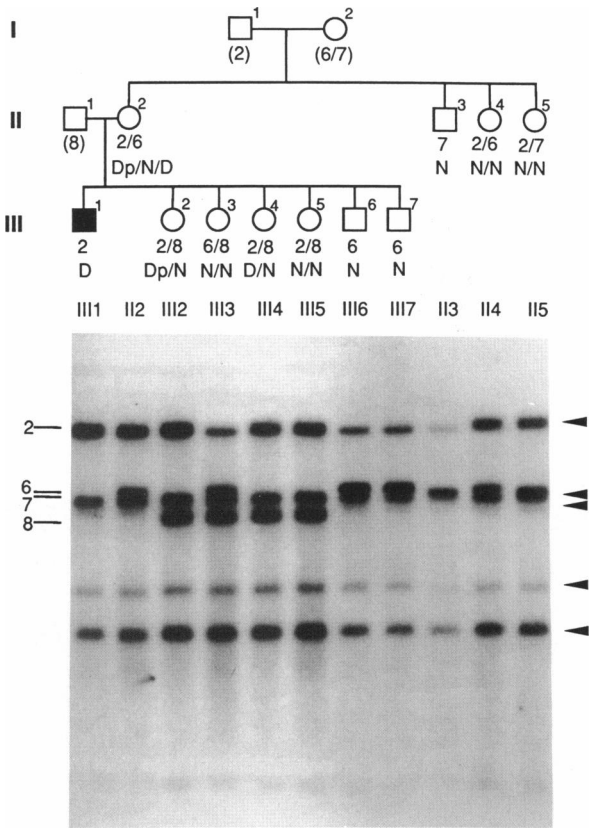

Figure 4 Pedigree analysis of the H96 family with the St14 probe. A Southern blot of *Taq*I-digested DNA from H96 family members was hybridized to the St14 probe. The polymorphic hybridizing fragments are indicated by allele numbers 2, 6, 7, and 8 as defined by Oberle et al. (1985). The five invariant hybridizing fragments are indicated by arrowheads; sizes are 5.5, 3.8, 3.65 (faint band), 2.2, and 1.7 kb. The relationship of the family members is indicated by the accompanying pedigree. Noted below the individual symbols of the pedigree are the St14 alleles and the nature of the factor VIII gene (N = normal; D = deleted; and Dp = duplicated). The parentheses indicate three individuals (father and grandparents) who were unavailable for study; their St14 alleles are inferred from the pedigree analysis.

curred first, and the deletion evolved from the duplication subsequently. The intron 22 sequences could have been duplicated in the grandpaternal gametes by either interchromatid exchange during meiosis or a transpositional event. Duplication during the mother's very early embryogenesis is also a possibility. Sequence analysis of the normal and duplicated genes does not reveal any homology between the inserted and target sequences and shows that the duplication is precise, with no bases being added or lost at the insertion breakpoint. One noteworthy feature of the duplicated intron 22 DNA is an adenine-rich track preceded by a polyadenylation signal sequence. Such a sequence is a hallmark of a processed retropseudogene (Weiner et al. 1986). Whether this sequence has any significance in the generation of the duplication is not known.

To continue with the model, the deletion may have resulted from intragenic recombination during the mother's embryogenesis, rendering her gametes—and, to a lesser extent, her peripheral blood leukocytes—mosaic. In peripheral blood, the vast majority of cells appear to be heterozygous for normal and duplicated genes. A small fraction of the cells contain the deleted chromosome and are probably heterozygous with the normal gene. The proportion of cells with deleted chromosomes appears to be higher in the germ line, since two of seven children have inherited the deletion. Although germ-line mosaicism has been described for deletions associated with Duchenne muscular dystrophy (Bakker, et al. 1987; Darras and Francke 1987; Lanman et al. 1987), there is no evidence for somatic mosaicism in these cases, nor is there evidence for an additional type of gene rearrangement in the mother.

The sequence at the deletion breakpoint was surprising. Figure 5 shows that the deletion occurred at a pair of 4-bp sequences CATT. These repeats are separated normally by 39 kb of DNA or, in the case of the duplication, by 62 kb. At the deletion boundary, both copies of CATT remain and are separated by a novel 9-bp sequence, resulting in direct repeat of 11 bp with a 2-bp spacer. Deletions at short repeat sequences have been observed during studies of *E. coli* deletion mutagenesis (Farabaugh et al. 1978) and the evolution of the globin gene family (Efstratiadis et al. 1980). However, in these deletion cases, thought to occur by a "slipped mispairing" mechanism, the repeat sequences are separated by several bases up to approximately 200 bp, only one copy of the repeat remains, and no stuffer DNA is inserted. A possible explanation for the generation of the deletion is intragenic homologous pairing of the 23-kb

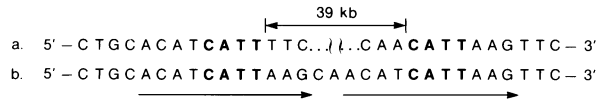

Figure 5 Sequence of DNA at the H96 deletion boundaries compared with normal sequence. Dotted lines in the normal DNA sequence corresponding to the 5′ and 3′ ends of the deletion breakpoint (*a*) represent the 39 kb that are deleted in patient H96. The deletion occurred at a 4-bp repeat sequence CATT and was accompanied by a small sequence insertion (*b*). The 11-bp direct repeat generated at the deletion boundary is underlined.

duplicated DNA. Such a "loop out" may have brought the CATT sequences in close proximity and allowed them to recombine. However, a recombination model would also predict that a single copy of CATT remain and would not account for the 11-bp repeat. Thus, the mechanisms that led to the duplication and deletion in this family appear to be more complex than simple transposition or recombination.

One interesting problem for genetic counseling is whether the partial gene duplication destroys factor VIII function. By coagulant activity and antigen measurements, both the mother and sister III-2 appear to have normal levels of factor VIII; the factor VIII:von Willebrand factor antigen ratios, determined by the method of Zimmerman et al. (1971), are normal values of 0.92 and 1.12 for the mother and sister, respectively. Since the duplicated sequence is entirely intron and has translocated to an intron, there would be no effect on the coding sequence if the exons continue to be properly spliced. However, given the probabilistic nature of the activity/antigen measurements for carrier diagnosis, and the possible instability of the duplication, prenatal diagnosis will be offered to the sister if desired.

Gene deletions are frequently associated with inherited diseases, but gene insertions and duplications are observed very rarely. For example, gene deletions are found in at least 50% of Duchene muscular dystrophy patients (Koenig et al. 1987). In hemophilia A, 5% of patients show deletions, but in over 600 patients examined, only two cases of insertions have been reported (Kazazian et al. 1988). The results presented here raise the possibility that duplications occasionally do occur but that they may not always be stably inherited. It will be interesting to see whether gene rearrangements accompany gene deletions in other sporadic incidences of X-linked disorders as more of these cases are analyzed.

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