Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3772–3776, June 1987
Genetics

Characterization of five partial deletions of the factor VIII gene

(hemophilia A/spontaneous mutation/inhibitor/carrier detection)

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Communicated by Victor A. McKusick, February 2, 1987

ABSTRACT **Hemophilia A is an X-linked disorder of coagulation caused by a deficiency of factor VIII. By using cloned DNA probes, we have characterized the following five different partial deletions of the factor VIII gene from a panel of 83 patients with hemophilia A: (*i*) a 7-kilobase (kb) deletion that eliminates exon 6; (*ii*) a 2.5-kb deletion that eliminates 5′ sequences of exon 14; (*iii*) a deletion of at least 7 kb that eliminates exons 24 and 25; (*iv*) a deletion of at least 16 kb that eliminates exons 23–25; and (*v*) a 5.5-kb deletion that eliminates exon 22. The first four deletions are associated with severe hemophilia A. By contrast, the last deletion is associated with moderate disease, possibly because of in-frame splicing from adjacent exons. None of those patients with partial gene deletions had circulating inhibitors to factor VIII. One deletion occurred *de novo* in a germ cell of the maternal grandmother, while a second deletion occurred in a germ cell of the maternal grandfather. These observations demonstrate that *de novo* deletions of X-linked genes can occur in either male or female gametes.**

Hemophilia A is a common disorder of blood coagulation caused by a deficiency of factor VIII (FVIII) (1, 2). The disorder is inherited as an X-linked recessive trait, and a significant proportion of the cases are due to *de novo* mutation (3). The clinical severity of hemophilia A correlates closely with the plasma concentration of FVIII, such that levels of <1% are associated with severe disease, and levels of 2–5% or greater are associated with moderate or mild disease (1). In addition, ≈10% of patients with severe disease develop antibodies against FVIII, called FVIII inhibitors, which usually appear after treatment with FVIII concentrates (4).

The gene for FVIII is 186 kilobases (kb) long and consists of 26 exons, with a mRNA sequence of ≈9 kb (5–8). An open reading frame encodes a protein of 2351 amino acids. An examination of intragenic homology with other proteins led to the characterization of three distinct regions, which are arranged in the order A1-A2-B-A3-C1-C2. The A domains share ≈30% sequence homology and the two C domains share 37% homology (5, 8). The function of these domains is largely unknown. To date, seven point mutations and three deletions have been identified in the FVIII gene of patients with hemophilia A (9–12).

In this study, we describe five additional deletions in different segments of the FVIII gene and examine their clinical consequence. We also show that two of these deletions occurred *de novo*, and we discuss the possible mechanisms by which they were produced.

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METHODS

Subjects. Our subjects were members of 83 families with hemophilia A who were referred for carrier detection and prenatal diagnosis. Four patients with deletions had FVIII levels of <1% as determined by an immunoradiometric assay (13), and the fifth patient (family E) had mild-to-moderate disease with FVIII levels of 2–5%. They ranged in age from 3 to 30 years, and none had developed FVIII inhibitors, although all severely affected patients had received FVIII concentrates for multiple bleeding episodes.

Restriction Endonuclease Analysis. Genomic DNA isolated from leukocytes was digested to completion with one of various restriction enzymes. Gel electrophoresis, transfer to nitrocellulose filters, hybridization, washing of filters, and autoradiography were performed as described (10, 12). The following cloned FVIII DNA fragments were used as probes: (*i*) probe A, a 1.7-kb *Kpn* I cDNA fragment that spans exons 1–12; (*ii*) probe BC, a 6.5-kb *Eco*RI cDNA fragment that spans exons 14–26; (*iii*) a 0.8-kb *Xba* I genomic fragment from the 3′ end of intron 13; and (*iv*) two genomic fragments from the 5′ region of intron 22, a 1.0-kb *Eco*RI/*Sst* I fragment, and a 1.6-kb *Bst*XI fragment, both of which can detect an *Xba* I polymorphic site in intron 22 (8, 10, 14). The first three probes were provided by J. Toole and J. Wozney (Genetics Institute, Boston), while the intron 22 probes were provided by R. Lawn (Genentech, South San Francisco, CA). The probes were labeled with [^{32}P]dCTP by nick-translation.

DNA Polymorphism Analysis. The following polymorphic sites within and closely linked to the FVIII gene were analyzed as described (5, 10, 15, 16): a *Bcl* I site 3′ to exon 18 of the FVIII gene; a *Bgl* I site 5′ to exon 26 of the FVIII gene; a *Bgl* II site detected with the anonymous probe DXS15; and *Taq* I site polymorphisms detected with the anonymous probe DXS52 (16). The latter two probes were kindly provided by K. Davies (Oxford, U.K.) and J. L. Mandel (Strasbourg, France).

RESULTS

Mapping the Deletions and Carrier Detection. After screening the genomic DNA of 83 patients with hemophilia A, we detected five deletions and eight point mutations within the FVIII gene (ref. 12; unpublished observations). Our screening strategy involved restriction analysis with *Taq* I, *Sst* I, and *Eco*RI, and successive hybridizations with probe A and probe BC.

Family A: *Taq* I digestion of genomic DNA from IV-1 (Fig. 1*a*) and hybridization with probe A showed the absence of a 2.4-kb fragment that would correspond to exons 5 and 6 (data not shown). The 5′ end was mapped by digestion with *Eco*RI, which showed absence of the 6.7-kb fragment (exon 6), and

Abbreviation: FVIII, factor VIII.

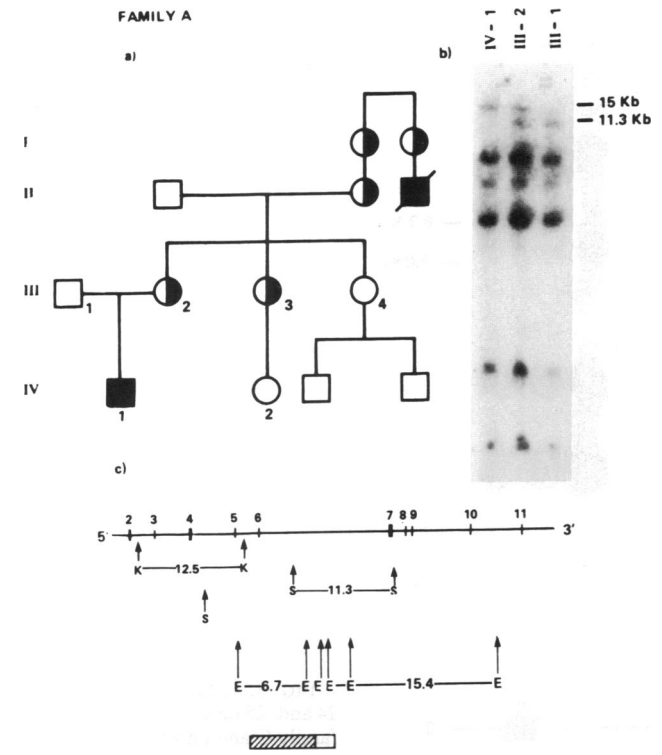

FIG. 1. Mapping of the exon 6 deletion. (*a*) Pedigree of family A (□, normal male; ■, hemophilic male; ○, normal female; ◑, carrier female). (*b*) Restriction analysis of DNA from members of family A cleaved with *Sst* I and hybridized with probe A. Family members are listed across the top of the autoradiogram. (*c*) Partial restriction map of the FVIII gene flanking the exon 6 deletion (E, *Eco*RI; K, *Kpn* I; S, *Sst* I; numbers between restriction sites indicate the size of fragments in kb). Hatched area below the map represents the minimum size of the deletion; open bar indicates uncertainty about the full extent of the deletion.

with *Kpn* I, which showed the normal 12.5-kb fragment (exons 3–5) (Fig. 1*c*). Thus, the 5′ breakpoint must lie 3′ to the *Kpn* I site in intron 5. *Eco*RI digestion also showed an intact 15.4-kb fragment (exons 7–10) (Fig. 1*c*), and *Sst* I digestion showed a new 15-kb junction fragment derived from exons 5 and 7, in place of the normal 11.3-kb fragment (Fig. 1 *b* and *c*). Thus, the deletion is limited to exon 6, and its 3′ end lies 5′ to the 15.4-kb *Eco*RI fragment within intron 6. A deletion of 7 kb is consistent with these observations. Subjects III-2 (Fig. 1*b*) and III-3 are carriers of the mutant gene, but IV-2 and III-4 are not (Fig. 1*a*).

Family B: Digestion of DNA from III-2 (Fig. 2*a*) with *Taq* I and hybridization with probe BC showed a new 9.2-kb DNA fragment and reduced hybridization at the 5.9-kb region (from exon 14) (Fig. 2 *b* and *d*). *Sst* I digestion showed absence of a 19.5-kb fragment, but an intact 3.2-kb fragment; and *Kpn* I digestion showed a new 11.5-kb fragment replacing the normal 7.5-kb fragment (Fig. 2 *c* and *d*). These results indicate that the deletion is limited to the sequences of exon 14 that lie 5′ to codon 926 (the *Sst* I site), and that it extends into intron 13. Hybridization with a genomic fragment from intron 13 (see *Methods*) showed the presence of the 3′ *Eco*RI site in that intron (Fig. 2*d*). Therefore, the size of this deletion is ≈2.5 kb. Subjects II-2 and III-1 are carriers of the mutant gene (Fig. 2 *b* and *c*).

In this pedigree, the deletion appeared to be a *de novo* mutation. This could not be confirmed directly because the parents of II-2 were deceased. However, we analyzed the DNA of two male and two female siblings of II-2 (Fig. 2*a*), none of whom were carriers of the mutant gene. Furthermore, the two female siblings (II-7 and II-8) shared a haplotype that was identical to the one bearing the mutant gene in II-2, indicating that the deletion appeared *de novo* in II-2. Comparison of these haplotypes with those of the male siblings (II-3 and II-4) indicated that the mutant gene originated in a germ cell of the maternal grandfather (I-1), who was 38 years old at the time of conception of his carrier daughter.

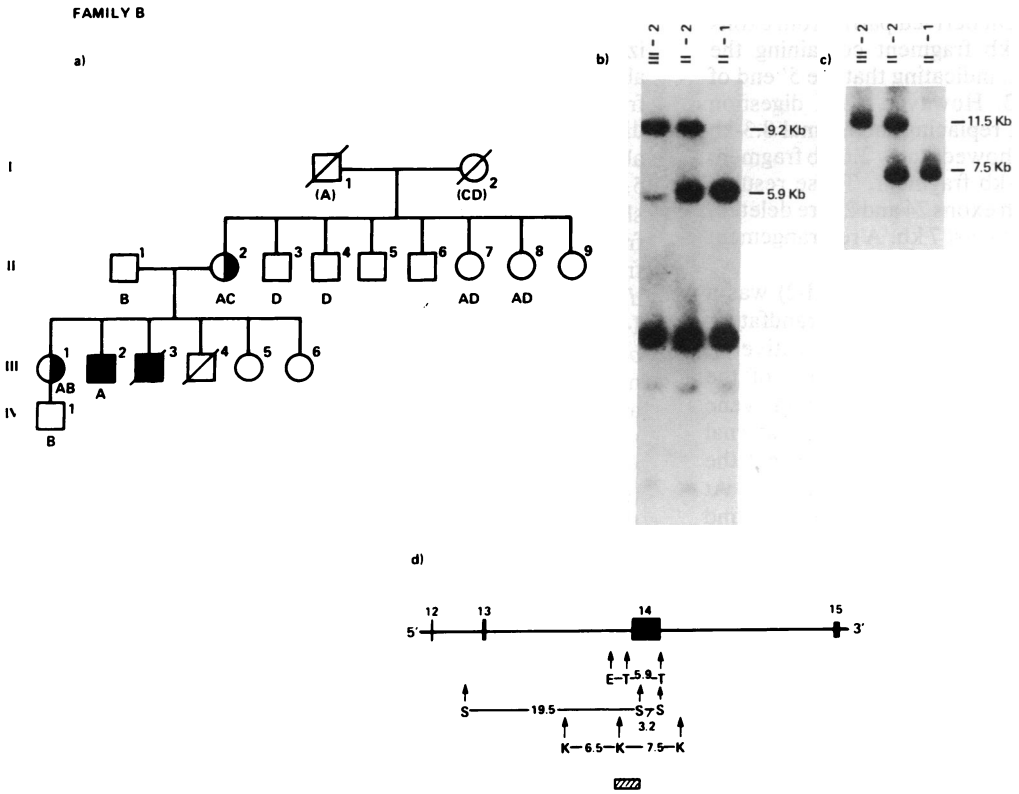

FIG. 2. Mapping of the exon-14 deletion. (*a*) Pedigree of family B. The letters below the subjects indicate chromosomal haplotypes. Haplotype designations: A, ++−1; B, ++−3; C, ++−2; D, −++3. These symbols represent DNA polymorphic markers in the following order: *Bcl* I, *Bgl* I, *Bgl* II, *Taq* I (see *Methods*). (*b*) Restriction analysis of DNA from members of family B cleaved with *Taq* I (T) and hybridized with probe BC. (*c*) *Kpn* I digestion of DNA from family B members hybridized with probe BC. (*d*) Partial restriction map and site of the exon-14 deletion. Symbols are the same as in Fig. 1.

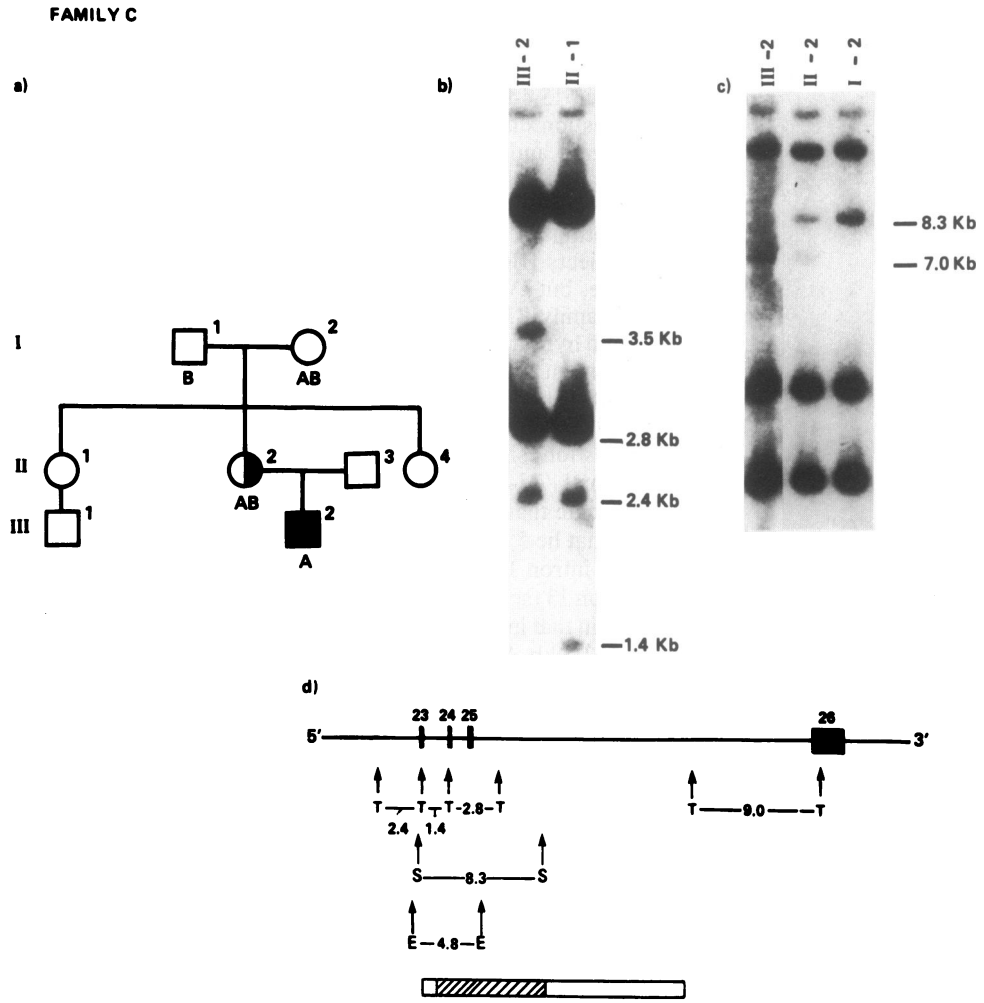

FIG. 3. Mapping of the exon-24 and -25 deletion. (*a*) Pedigree of family C and chromosomal haplotypes: A, −−+; B, +++. Symbols are in the same order as in Fig. 2. (*b* and *c*) Restriction analysis of DNA from members of family C cleaved with *Taq* I (*b*) and *Sst* I (*c*), and hybridized with probe BC. (*d*) Partial restriction map and site of the exon-24 and -25 deletion. Symbols are the same as in Fig. 1.

Family C: *Taq* I digestion of DNA from III-2 (Fig. 3*a*) and hybridization with probe BC revealed a new 3.5-kb fragment and absence of the 1.4-kb fragment derived partly from exons 23 and 24 (Fig. 3*b*). The 2.4-kb fragment containing the remainder of exon 23 was intact, indicating that the 5′ end of the deletion is within intron 23. However, *Sst* I digestion revealed a new 7.0-kb fragment replacing the normal 8.3-kb fragment, and *Eco*RI digestion showed a new 2.0-kb fragment and absence of the normal 4.8-kb fragment. These results, taken together, indicate that both exons 24 and 25 are deleted. The total size of the deletion is at least 7 kb. A rearrangement at the 3′ breakpoint cannot be excluded.

By restriction analysis, the patient's mother (II-2) was a carrier of the mutant gene, but not the maternal grandfather or grandmother (Fig. 3 *a* and *c*; I-1 and I-2, respectively). Therefore, the mutation appeared *de novo* in one of the mother's X chromosomes. Furthermore, the mutant gene was associated with the haplotype derived from the maternal grandmother (Fig. 3*a*; data not shown), indicating that the mutation arose in an ovum of the maternal grandmother. At the time of conception of II-2, the ages of her father (I-1) and mother (I-2) were 29 and 27 years, respectively.

Family D: *Sst* I digestion of DNA from IV-1 (Fig. 4 *a* and *b*) and hybridization with probe BC showed the absence of a normal 8.3-kb fragment (exons 23–25). Therefore, the deletion includes exons 23–25. The 5′ end was mapped using a 1.6-kb genomic fragment from intron 22 as probe. Hybridization of *Sst* I-digested DNA from IV-1 with this probe showed absence of the normal 18.5-kb fragment and presence of a new 15.0-kb fragment (Fig. 4*c*), indicating that the deletion extends beyond the *Sst* I site 5 kb into intron 22. The 3′ end of the deletion does not extend beyond a *Taq* I site that is ≈9 kb 5′ to exon 26. The minimum deletion size is 16 kb.

Family E: *Taq* I digestion of DNA from III-1 and hybridization with probe BC showed a new 4.0-kb fragment and absence of a 5.9-kb fragment (exons 20–22); the 2.2-kb fragment (exons 18 and 19) was intact (Fig. 5 *b* and *d*). *Eco*RI digestion showed an intact 1.4-kb fragment (exons 19 and 20), absence of a 5.3-kb fragment (exons 21 and 22), but a new 5.8-kb fragment (Fig. 5*d*; data not shown). *Sst* I digestion showed a new 15-kb fragment replacing the normal 12.5-kb fragment, and *Bam*HI digestion showed a new 10.5-kb fragment replacing the normal 9.9-kb fragment (Fig. 5 *c* and *d*). The 3′ breakpoint was mapped by hybridization of the *Sst* I-cleaved DNA with the 1.6-kb intron 22 probe (see *Methods*), which showed a normal 18.5-kb fragment (Fig. 5*d*; data not shown). Digestion of DNA from III-2, with both *Xba* I and *Kpn* I and hybridization with the 1.0-kb intron 22 probe, showed that she was heterozygous at the *Xba* I site, and there was no alteration in the size of the 4.8-kb or the 6.2-kb alleles (data not shown). This result placed the 3′ breakpoint within the 4.8-kb *Bam*HI fragment (Fig. 5*d*). These data are consistent with a 5.5-kb deletion involving exon 22.

DISCUSSION

Several molecular defects have been identified within the FVIII gene in patients with hemophilia A. These consist of three different partial deletions and six point mutations that generate nonsense codons (9–12), including two different spontaneous recurrences of nonsense mutations (12), and a seventh point mutation that leads to an amino acid substitu-

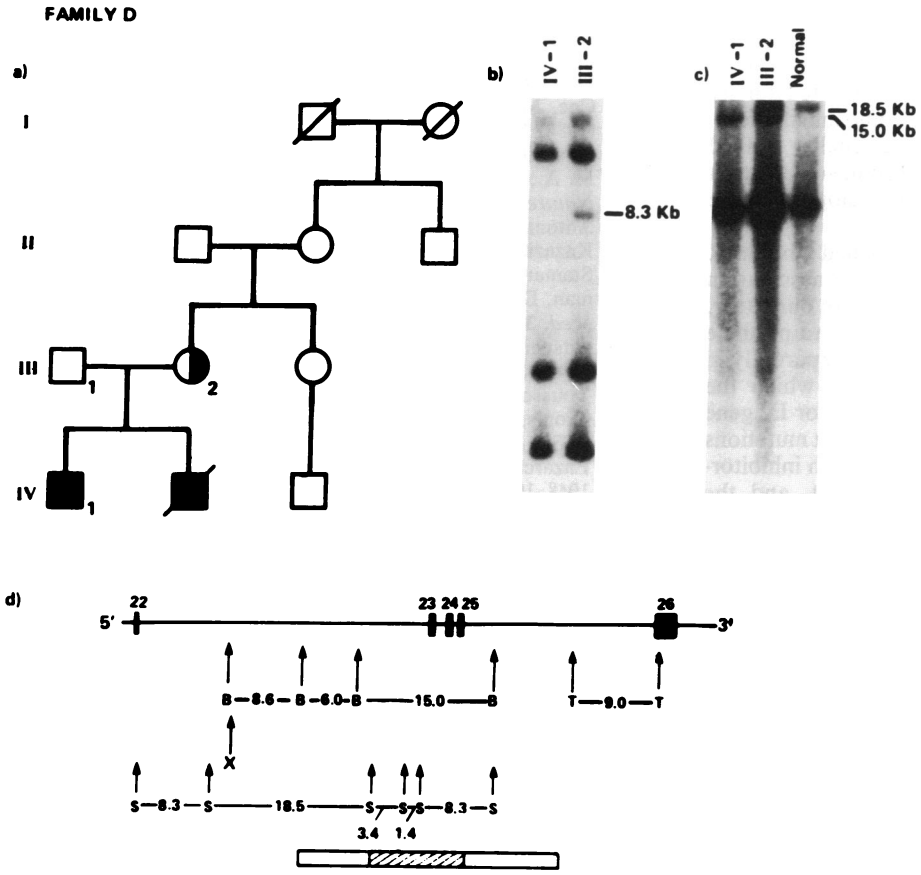

FIG. 4. Mapping of the exon-23 to -25 deletion. (*a*) Pedigree of family D. (*b* and *c*) Restriction analysis of DNA from members of family D cleaved with *Sst* I and hybridized with probe BC (*b*), and with the 1.6-kb probe from intron 22 (*c*). (*d*) Partial restriction map and site of the exon-23 to -25 deletion. B, *Bam*HI; T, *Taq* I; X, *Xba* I polymorphic site. Other symbols are the same as in Fig. 1. The parents of III-2 were not available for study.

tion (11). With the exception of the latter mutation, all were associated with severe disease.

In this study, we report five deletions from a population of 83 patients (6% prevalence) that allow us to define better the topography of the FVIII gene. An examination of the nucleotide sequence reported for the coding region of FVIII (5, 6) reveals that both the exon-14 and exon-24 and -25 deletions lead to frameshifts. By contrast, the exon-6, exon-22, and exon-23 to -25 deletions probably cause in-frame mutations that delete 38 amino acids (A1 domain), 52 amino acids (C1 domain), and 157 amino acids (C1 and C2 domains), respectively. These outcomes assume correct splicing of the adjacent exons, which has been documented in several other systems, including simian virus 40–β-globin recombinant constructs (17) and α1 type I collagen (18). Thus, selective deletion of sequences from the C1 domain may generate a molecule of moderate functional activity.

In three of the families, inspection of the pedigrees suggested *de novo* mutation. We could show this conclusively in family C, and with very high likelihood in family B. We cannot rule out the slim possibility that the mutation in subject II-2 (family B) was inherited from a hemophilic father who was not the stated father, but carried the identical FVIII chromosomal haplotype as the stated father. Haldane postulated that one-third of the mutations in X-linked "lethal" disorders represent *de novo* events (3). Families B and C demonstrate *de novo* deletion in this disease.

Because of the difference in timing of gametogenesis in males and females, it was believed that mutations arising on paternal X chromosomes constitute almost exclusively point mutations (19, 20). Mutations of maternal X-linked genes would also include deletions (20), presumably due to errors of recombination. Our finding of the origin of a gene deletion in a germ cell of a hemizygous male shows that this cannot be the sole explanation. Studies of deletions in the β-globin gene cluster (21, 22) and in the gene for the low density lipoprotein

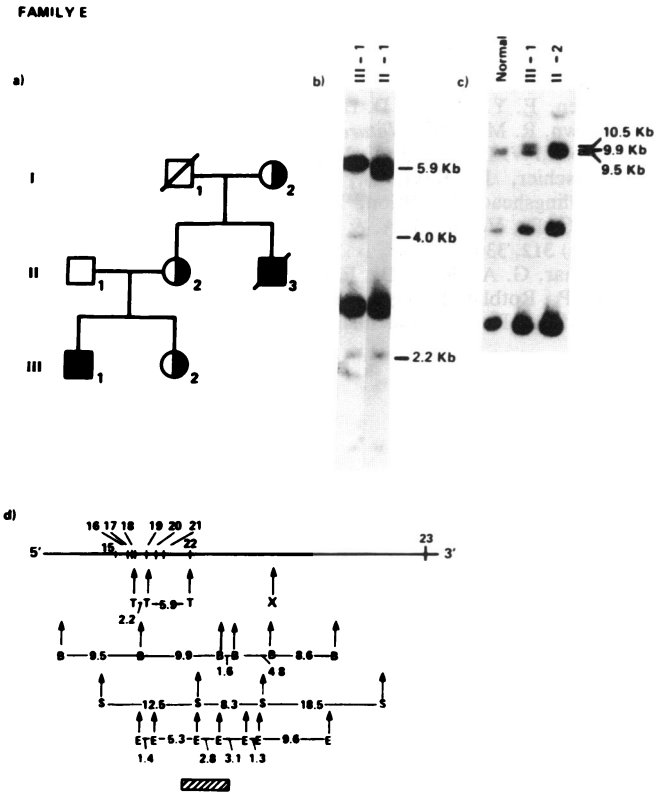

FIG. 5. Mapping of the exon-22 deletion. (*a*) Pedigree of family E. (*b* and *c*) Restriction analysis of DNA from members of family E cleaved with *Taq* I (*b*) or *Bam*HI (*c*), and hybridized with probe BC. (*d*) Partial restriction map and extent of the exon-22 deletion. Symbols are the same as in Figs. 1 and 4.

receptor (23, 24) suggest that local misalignment between short stretches of DNA sequence homology may be an important feature. Thus, in our study, while the maternally derived exon-24 and -25 deletion can be accounted for by either an intrachromosomal or an interchromosomal event, the most plausible explanation for the paternally derived exon-14 deletion appears to be an intrachromosomal event leading to breakage and reunion, possibly analogous to the examples stated earlier (21–24).

Finally, none of our patients with deletions had inhibitors. Several investigators have addressed the possible association of inhibitors with gene deletions (9, 10, 25–27). In our study, partial gene deletions *per se* were not sufficient to produce the "inhibitor" phenotype. These findings are apparently in contrast to the situation in hemophilia B, in which the majority of patients with inhibitors have had factor IX gene deletions (25–27). In hemophilia A, nonsense point mutations in the FVIII gene have also been observed in both inhibitor-positive and inhibitor-negative patients (9, 10), and the frequency of the inhibitor phenotype in patients with gene deletions may be no greater than in patients with nonsense mutations (unpublished data). Thus, although the molecular basis of inhibitor production in hemophilia A remains elusive (27), gross deletions of the FVIII gene do not appear to play a major role in its pathogenesis.

We thank Dr. V. A. McKusick for a critical reading of the manuscript, Dr. D. Broome for providing blood samples, and Ms. E. Pasterfield for expert preparation of the manuscript. This study was supported by National Institutes of Health grants to S.E.A. and H.H.K.

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