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Genetics

Molecular characterization of severe hemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene

(polymerase chain reaction/denaturing gradient gel electrophoresis)

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ABSTRACT **Hemophilia A is an X chromosome-linked disorder resulting from deficiency of factor VIII, an important protein in blood coagulation. A large number of disease-producing mutations have been reported in the factor VIII gene. However, a comprehensive analysis of the mutations has been difficult because of the large gene size, its many scattered exons, and the high frequency of *de novo* mutations. Recently, we have shown that nearly all mutations resulting in mild-to-moderate hemophilia A can be detected by PCR and denaturing gradient gel electrophoresis (DGGE). In this study, we attempted to discover the mutations causing severe hemophilia A by analyzing 47 unselected patients, 30 of whom had severe hemophilia and 17 of whom had mild-to-moderate disease. Using DGGE as a screening method, we analyzed 99% of the coding region, 94% of the splice junctions, the promoter region, and the polyadenylylation site of the gene. We found the mutation in 16 of 17 (94%) patients with mild-to-moderate disease but in only 16 of 30 (53%) patients with severe hemophilia A. Since DGGE after computer analysis appears to detect all mutations in a given fragment, the lower-than-expected yield of mutations in patients with severe disease is likely not due to failure of the detection method; it is probably due to the presence of mutations in DNA sequences outside the regions studied. Such sequences may include locus-controlling regions, other sequences within introns or outside the gene that are important for its expression, or another gene involved in factor VIII expression that is very closely linked to the factor VIII gene.**

Hemophilia A is a common hereditary disorder of blood coagulation resulting from deficiency of factor VIII. Factor VIII is a plasma glycoprotein that after posttranslational modification plays an important role in the intrinsic coagulation pathway (1, 2). Patients with hemophilia A are classified according to clinical severity and the activity of factor VIII in their plasma. In severe, moderate, and mild hemophilia A, factor VIII activity is <1%, 1–5%, and 5–30%, respectively. The factor VIII gene has been cloned (3, 4) and mapped to the distal long arm of the X chromosome (5). A large number of mutations in the factor VIII gene that cause hemophilia A have been reported (6–15). The factor VIII gene is 186 kilobases and contains 26 exons. It is difficult to characterize all possible mutations in patients both because of the large size of the gene and because different mutations exist in unrelated patients due to the high frequency of *de novo* mutations.

A number of screening methods have been developed to detect point mutations, including direct genomic sequencing (10, 16), chemical (17) or enzymatic cleavage (18, 19) of nucleotide mismatches, discriminant oligonucleotide hybridization (7, 14), denaturing gradient gel electrophoresis (DGGE) (11, 12, 19–21), and single-strand gel electrophoresis (SSGE) (22). All of these methods are used after PCR amplification.

Recently, we have used DGGE analysis to detect all possible mutations in 29 patients with mild-to-moderate hemophilia A. DGGE analysis was an excellent method since the disease-producing mutation was found in 25 of these patients (15).

In this report, we used a similar analysis in 47 unselected patients with hemophilia A, 30 of whom had severe disease. Our goal was to compare the nature of mutations in severe hemophiliacs with those in patients with mild-to-moderate disease. The disease-producing mutation was found in nearly all patients with mild-to-moderate hemophilia A (16 of 17); however, the mutation was found in only 16 of 30 patients with severe disease.

MATERIALS AND METHODS

Subjects. We studied 47 unselected patients with hemophilia A whose mutations in the factor VIII gene were unknown. Of these patients, 30 had severe hemophilia A and 17 had mild-to-moderate disease. All the patients with severe disease have factor VIII activity of ≤1%, while mild-to-moderate patients have factor VIII activity ranging from 2% to 29%. Two of the 30 patients with severe hemophilia A developed antibodies against factor VIII after transfusion therapy.

Southern Blot Analysis. Restriction endonuclease analysis was performed as described elsewhere (23) using *Taq* I and three subfragments of factor VIII cDNA as hybridization probes (4, 23).

Amplification of Genomic DNA. Amplification of high molecular weight genomic DNA from leukocytes was performed by PCR (15, 24). The entire coding region, 47 of 50 splice junctions, promoter region including the TATA box, and 150 nucleotides 5′ to the cap site and 345 nucleotides from the 3′ untranslated and flanking region including the polyadenylylation site of factor VIII gene were amplified by using 47 pairs of oligonucleotide primers. A 40-nucleotide (nt) G+C-rich

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; SSGE, single-strand gel electrophoresis; nt, nucleotide(s); IVS, intervening sequence.

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sequence (G+C clamp) was attached to either the 5′ or the 3′ primer (15, 25). Computer analysis was carried out by using the programs MELT87 and SQHTX provided by L. Lerman (Massachusetts Institute of Technology, Cambridge) to aid the choice of PCR primers (15, 26). Forty-five sets of primers were described elsewhere (15); the two additional sets used here were (*i*) 5′-(GC)x-GCTCCTGTTCACTTTGACTT-3′ and 5′-AGAGCTCTATTTGCATGACTTA-3′ for the promoter region containing the probable TATA box and the 5′ mRNA start site (3) and (*ii*) 5′-ATAGCCCGGTAGAGGAGTTAAC-3′ and 5′-(GC)x-CATTTCTGATTTATTTTATTCAGAC-3′ for the polyadenylylation site. (GC)x indicates a 40-nt G+C clamp (15). Amplification was performed with 200–400 ng of genomic DNA template, 400 nM each primer, 200 μM each dNTP and 2 units of *Taq* polymerase per 100-μl reaction mixture for 35 cycles (at 94°C for 20 sec, thermal transition from 94°C to the annealing temperature over 2 min, 52°C–55°C for 45 sec, and 72°C for 30–60 sec).

DGGE and Sequence Analysis. DGGE was performed under conditions determined empirically for a given PCR product as described (19, 21). Approximately 80 ng of PCR product from each of two patients was combined to form heteroduplexes. Electrophoresis was performed on a 6.5% polyacrylamide gel (14 cm × 19 cm × 0.75 mm) containing a linear gradient of denaturants at 2–5 V/cm for 16–23 hr. DNA fragments were visualized after staining with ethidium bromide. PCR products showing abnormal migrating patterns were directly sequenced (15, 16).

DNA Polymorphism Analysis. Amplification of genomic DNA and subsequent restriction analysis of PCR products were performed as described (15, 27). Factor VIII haplotypes were derived for six polymorphic sites in the factor VIII gene: intervening sequence 7 (IVS7), nt 27 (11, 12); exon 14, codon 1421 (3, 15); exon 14, codon 1488 (15); IVS*18*, *Bcl* I site (27); IVS*22*, *Xba* I site (28); a G to A nucleotide substitution, 110 nt 5′ to the polyadenylylation site, first found as a nucleotide difference between cloned cDNA and genomic DNA (3). The frequency of the latter site was determined by DGGE. Haplotypes at the remaining sites were determined as described (15, 27).

RESULTS

We used two different methods to screen for mutations in 47 patients with hemophilia A. First, DGGE analysis of amplified products was carried out to detect nucleotide substitutions and insertion–deletion of a few nucleotides (6). An example of DGGE screening for mutations is shown in Fig. 1. Second, we used Southern blot analysis to identify gross factor VIII gene rearrangements.

Table 1 lists all mutations identified, and Fig. 2 shows these mutations schematically. A total of 30 point mutations and 2 partial gene deletions were found. The 30 point mutations included 25 single nucleotide substitutions, deletions of 2 and 23 nt, and 3 insertions of 1 nt each. The gross gene alterations included a deletion of exon 1 and a deletion of exons 15–21. All mutations were detected by DGGE analysis or absence of

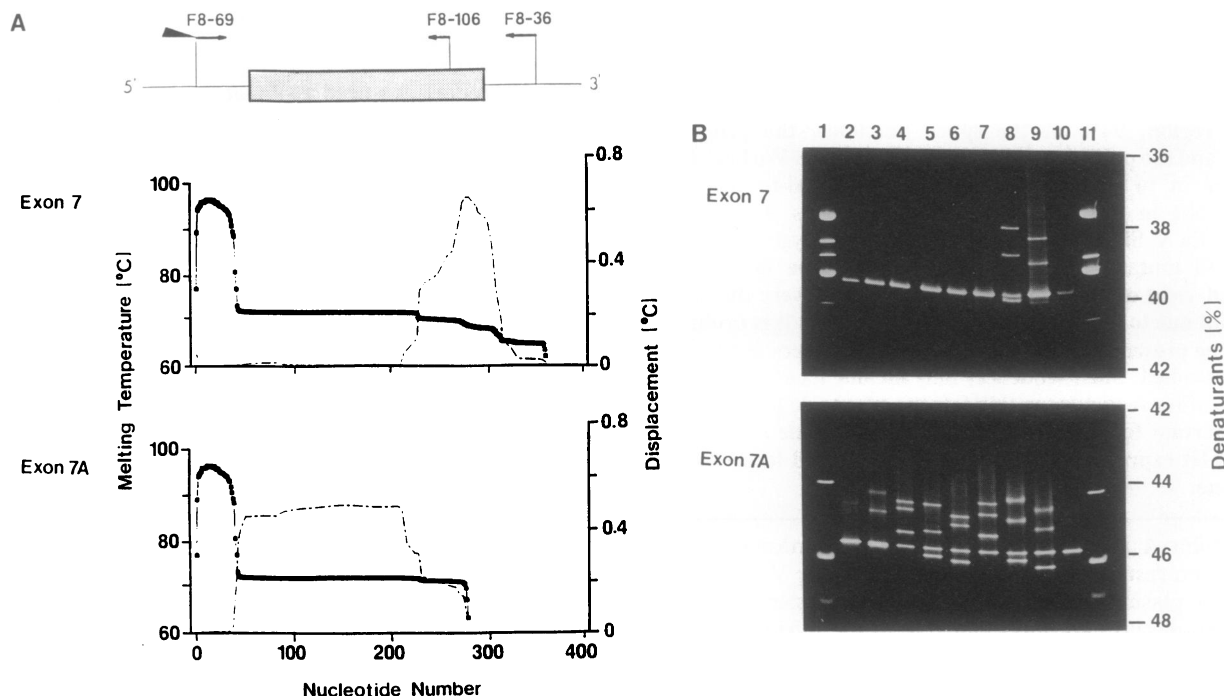

FIG. 1. Computer analysis and mutation detection of the exon 7 region by DGGE. (*A*) (*Upper*) Map of exon 7 region and location of oligonucleotide primers for PCR amplification. A 358-base-pair (bp) exon 7 fragment and a 277-bp exon 7A fragment were amplified using primer sets F8-69 and F8-36, and F8-69 and F8-106, respectively. The primer, F8-69, contained a 40-bp G+C clamp shown by thick arrowhead. The melting temperature maps and the displacement maps of the exon 7 fragment (*Middle*) and 7A fragment (*Lower*) are shown. The melting temperature map was calculated by using the program MELT87, while the displacement map was generated by using the program SQHTX (26). Solid lines represent the calculated melting temperature at which each base pair has a 50% probability of being in a melted state (melting map). Dashed lines represent the calculated difference in the melting temperature at the optimal running time between the homoduplex of normal DNA and the heteroduplex containing a mismatch due to a single nucleotide substitution (displacement map). (*B*) DGGE analysis of exon 7 fragments. PCR products containing known and newly discovered mutations were combined with PCR products from a normal male to form heteroduplexes and subjected to DGGE. PCR products were electrophoresed in a 30–50% denaturing gradient gel for 18 hr at 40 V for exon 7 (*Upper*) and in a 35–55% denaturing gradient gel for 18 hr at 60 V for exon 7A (*Lower*). Lanes: 1 and 11, OX174 DNA digested with *Hae* III; 2 and 10, normal male; 3, G → C position 1 in IVS*6*; 4, G → A codon 274; 5, T → G codon 285 (JH-85); 6, A → G codon 291 (29); 7, G → A codon 301 (JH-86); 8, T → C codon 301 (JH-62); 9, A → G codon 314 (15). As predicted from the computer analysis, mutations that occur within the 5′ two-thirds of exon 7 and IVS*6* were not detected when the exon 7 fragment was analyzed, because these mutations do not reside within the first melting domain of the exon 7 fragment. However, these mutations were distinguishable from normal when the exon 7A fragment was analyzed because they are located within the first melting domain of the latter fragment.

Table 1. Summary of mutations identified

Patient	Severity of hemophilia A	Exon	Codon(s)	Nucleotide change	Structural change	Domain	Transition at CpG	Haplotype
JH-73	M	3	108	AAG → ACG	Lys-89 to Thr	A_1	No	1
JH-71	M	3	110	ATG → GTC	Met-91 to Val	A_1	No	4
JH-72	S	3/IVS3	Deletion of 23 nt*					1
JH-85	M	7	285	GTG → GGG	Val-266 to Gly	A_1	No	2
JH-86	S	7	301	CGC → CAC	Arg-282 to His	A_1	Yes	4
JH-62	M	7	312	TTC → TCC	Phe-293 to Ser	A_1	No	1
JH-74	S	9	444	AAA → AGA	Lys-425 to Arg	A_2	No	2
JH-70	M	10	492	TAT → CAT	Tyr-473 to His	A_2	No	1
JH-63	S	11	561	GAT → GGT	Asp-542 to Gly	A_2	No	1
JH-82/-83/-84	M	12	612	CGC → TGC	Arg-593 to Cys†	A_2	Yes	4/4/4
JH-78/-79	M	14	723	GCC → ACC	Ala-704 to Thr	A_2	Yes	1/5
JH-77	S	14	1414	TCA → TCAA	Frameshift	B		1 or 2
JH-81	S	14	1458–1460	$A_8 \rightarrow A_9$	Frameshift	B		1
JH-80	S	14	1555/1556	TGGAAT → TGAT	Frameshift	B		4
JH-52	M	14	1699	TAT → TTT	Tyr-1680 to Phe‡	A_3	No	1
JH-128	M	16	1800	CGT → CAT	Arg-1781 to His	A_3	Yes	1
JH-127	S	16	1803	TCC → TAC	Ser-1784 to Tyr	A_3	No	1
JH-129	S	17	1907	$A_4 \rightarrow A_5$	Frameshift	A_3		1
JH-56	M	18	1941	AAT → GAT	Asn-1922 to Asp§	A_3	No	4
JH-55	S	18	1941	AAT → AGT	Asn-1922 to Ser	A_3	No	4
JH-60	S	23	2166	CGA → TGA	Arg-2147 to stop¶	C_1	Yes	1
JH-57/-58/-59	M	23	2169	CGT → CAT	Arg-2150 to His‖	C_1	Yes	2/4/2
JH-64	S	24	2228	CGA → CAA	Arg-2209 to Gln**	C_2	Yes	1
JH-54	S	26	2323	CGC → TGC	Arg-2304 to Cys	C_2	Yes	2
JH-53	S	26	2326	CGA → TGA	Arg-2307 to stop††	C_2	Yes	1
JH-145	S	1		Deletion of exon 1		A_1		1
JH-141	S	15–21		Deletion of exons 15–21		A_3		3A

M, mild-to-moderate hemophilia A; S, severe hemophilia A. Haplotypes were determined at the following polymorphic sites: IVS*7*, nt 27; codon 1421; codon 1488; *Bcl* I site in IVS*18*; *Xba* I site in IVS*22*; 3′ untranslated region. Haplotype 1, G,C,A,+,+,A; haplotype 2, G,C,A,+,−,A; haplotype 3, G,C,A,−,−,G; haplotype 3A, G,C,A,del,del,G; haplotype 4, A,G,A,−,−,G; haplotype 5, G,C,C,−,−,G.

*This deletion included the IVS*3* splice donor site and resulted in a frameshift.

†An additional nucleotide substitution eliminating a *Taq* I site either in IVS*4* or in IVS*6* was identified in all three patients.

‡Similar mutation has been reported in a different patient in ref. 10.

§Similar mutation has been reported in a different patient in ref. 12.

¶Similar mutation has been reported in a different patient in ref. 30.

‖Similar mutation has been reported in a different patient in ref. 15.

**Similar mutation has been reported in a different patient in ref. 31.

††Similar mutation has been reported in a different patient in ref. 32.

amplification by PCR; furthermore, the two large deletions and two nucleotide substitutions that alter *Taq* I sites were also detected by Southern blot analysis. In addition, a rare *Taq* I site DNA polymorphism either in IVS*4* or in IVS*6* was identified in three patients (JH-82, JH-83, JH-84). The remaining 29 patients listed in Table 1 had only one mutation in the DNA regions studied.

Our analysis included 47 regions of the factor VIII gene containing 99% of the coding region, 47 of 50 splice junctions, the promoter region and the polyadenylylation site region. The mutation was not identified in 15 patients—14 of 30 (47%) patients with severe hemophilia A and 1 of 17 (6%) patients with mild-to-moderate disease.

Among 25 single nucleotide substitutions, 23 were missense and 2 were nonsense mutations. Three different mutations were found in more than 1 patient (see Table 1) and, therefore, the total number of distinct molecular lesions was 27. Six of these 27 defects have been described (see Table 1 for references). A considerable number of mutations occur in CG dinucleotides conforming to the CG to TG "mutation hot spot" rule. These included 14 of 32 (44%) total mutations or 14 of 25 (56%) single nucleotide substitutions.

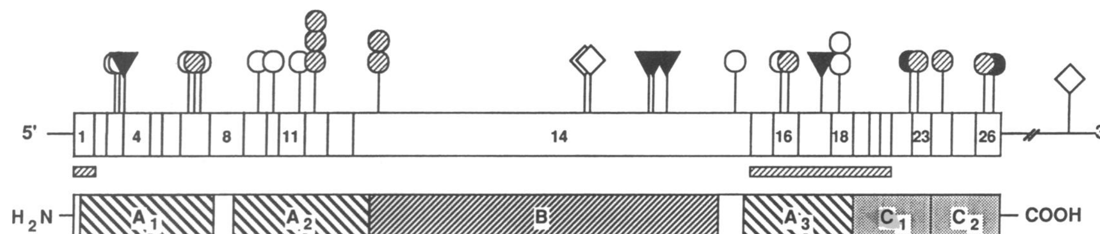

FIG. 2. Location of sequence changes identified in this study. Open bar shows the coding region of factor VIII cDNA with its 26 exons (selected exons are numbered). Two large deletions are shown as hatched bars. The domain structure of factor VIII protein is shown below. The open boxes between domains A_1 and A_2 and between B and A_3 are the two acidic regions of the protein. The locations of single nucleotide substitutions are shown by circles. Hatched circles (missense mutations) and solid circles (nonsense mutations) indicate mutations at CpG dinucleotides. Solid triangles denote microdeletions and microinsertions, and open diamonds denote neutral polymorphisms.

The G or A nucleotide polymorphism 110 nt 5′ to the polyadenylylation site (3) had the following frequency: G was present in 32% and A was present in 68% of X chromosomes studied. This polymorphism is in complete linkage disequilibrium with five other polymorphic sites. Haplotypes for these six sites are presented in Table 1.

DISCUSSION

Differences in Mutation Detection Between Severe and Mild-to-Moderate Hemophilia A Patients. In this study, we used DGGE to search for molecular defects in the factor VIII gene in two groups of patients with hemophilia A. The mutations were found in almost all patients with mild-to-moderate hemophilia but in only about 50% of the patients with severe hemophilia A (Table 2).

We selected DGGE as a screening method because we had concluded that DGGE after computer analysis could detect nearly all point mutations. Previously, we were able to identify mutations in 25 of the 29 patients with mild-to-moderate hemophilia A (15) (Table 2). Moreover, in that study 26 other known point mutations in the factor VIII gene were analyzed, and all were distinguishable from normal by this method (15).

In the present study, mutation detection in patients with mild-to-moderate hemophilia A was, as expected, nearly complete; however, detection of mutations in severe disease was only 53%. In contrast, in severe hemophilia B due to factor IX deficiency, the causative mutation was detected in the coding region of the factor IX gene in 17 of 17 patients analyzed (33). Four explanations of the low yield of mutations in severe hemophilia A may be entertained.

(*i*) DGGE analysis is not an optimal method to detect deletions or insertions of a few nucleotides. However, this is unlikely because three known frameshift mutations including deletions of 2 and 3 nt and insertion of 1 nt were detected by DGGE. Furthermore, in this study we identified four frameshift mutations due to insertion of 1 nt in three cases and deletion of 2 nt in one case. The number of frameshift mutations identified in this study [4 in 30 (13%) severe hemophilia A patients] is not different from that found in patients with severe hemophilia B [2 of 17 (12%)] (33) or from that in Lesch–Nyhan syndrome due to deficiency of hypoxanthine phosphoribosyltransferase [8 of 48 (17%)] (34). To be certain that no mutation escaped detection after DGGE analysis, we sequenced three selected regions of the factor VIII gene in all 47 patients—namely, exons 6, 15, and 19 and their splice junctions. No mutations were found in these regions. Furthermore, comparison of DGGE and SSGE in a total of 160 patients with hemophilia A showed that there were no mutations detected by SSGE that were not also detected by DGGE; in contrast, some DGGE-detected mutations were not found by SSGE.

(*ii*) The mutations are in areas of the factor VIII gene that were not analyzed. These areas include intervening sequences outside of splice junctions, most of the 3′ untranslated region, and unknown promoter or locus-activating regions. It is unlikely that many mutations in introns cause severe hemophilia, since only 5 of ≈100 mutations in the β-globin gene that produce β-thalassemia are internal intron mutations, and only 2 of these produce β^0-thalassemia (35). No known mutations in the 3′ untranslated region of genes produce severe phenotypes. All 12 known point mutations in the promoter region of the β-globin gene produce β^+-thalassemia (35). On the other hand, a rare *de novo* deletion of the locus-activating region of the β-globin gene cluster completely inhibits the expression of the β-globin gene (36). If most unknown mutations in severe hemophilia A occur in a locus-activating region, then this region is either several kilobases long or it contains a hot spot for mutations—e.g., a region susceptible to deletion by unequal crossover due to direct repeats.

(*iii*) The mutations are present in another gene on the X chromosome, either within the factor VIII gene or very closely linked to it. In addition, mutations in this hypothetical gene would have to completely eliminate expression of the factor VIII gene.

(*iv*) Abnormal methylation of the factor VIII gene and its neighboring sequences may be associated with total inactivation of the factor VIII gene in certain patients. No such example has been described to date in a mammalian gene system. However, recent reports of differential methylation close to the fragile Xq27.3 site in patients with the fragile X syndrome (37, 38) suggest this possibility.

Factor VIII Mutations of Interest. Of the 32 mutations identified in this study, 7 missense mutations are associated with severe hemophilia A—Arg-282 to His, Lys-425 to Arg, Asp-542 to Gly, Ser-1784 to Tyr, Asn-1922 to Ser, Arg-2209 to Gln, and Arg-2304 to Cys. Thus, these amino acid residues are important for processing and function of factor VIII protein. Two unrelated patients had different missense mutations at Asn-1922 (Asn-1922 to Ser in JH-55 and Asn-1922 to Asp in JH-56). We have previously identified an additional patient, JH-51, with an Asn-1922 to Asp substitution (12). The mutation in JH-56 occurred *de novo* in the maternal germ cells. These results indicate that Asn-1922 is essential for normal factor VIII expression. Moreover, this amino acid is conserved in all three A domains of both factors V and VIII. Two different missense mutations were also found at the Tyr-473 residue in mild-to-moderate patients. A Tyr-473 to Cys substitution was previously identified (15). In this study, we identified a Tyr-473 to His substitution, indicating that this amino acid residue is of functional importance.

Table 2. Mutation detection by DGGE

Mutation	Unselected patients (n = 47): Severe (n = 30)	Unselected patients (n = 47): Mild-to-moderate (n = 17)	Patients with mild-to-moderate hemophilia A* (n = 29)	Patients with mild-to-moderate hemophilia A combined† (n = 46)
Known				
Missense	7 (23%)	16 (94%)	24 (83%)	40 (87%)
Other	9‡ (30%)	0	1§ (4%)	1 (2%)
Unknown	14 (47%)	1 (6%)	4 (14%)	5 (11%)

*Data from ref. 15.
†This group consists of 17 patients from the unselected group (in this study) and 29 patients selected for mild-to-moderate hemophilia A (15).
‡Four frameshift mutations, two nonsense mutations, two large partial gene deletions and one small deletion of 23 nt.
§Splicing mutation.

The Ala-704 to Thr mutation due to a G to A change at a CpG dinucleotide was found in two unrelated patients (JH-78 and JH-79) with two different factor VIII haplotypes. The mutation in JH-78 occurred *de novo* in his mother; therefore, this suggests that the Ala-704 to Thr substitution in the A_2 domain is the disease-producing mutation in this patient, although the amino acid substitution does not seem to be dramatic.

JH-81 with severe hemophilia A had an insertion of an A residue within eight consecutive A residues in exon 14. Usually, this type of mutation is due to slippage in replication (39). Interestingly, in another group of hemophilia A patients, we found one case with deletion of an A residue within the same stretch of eight A residues (15). Both mutations were detected by DGGE.

The coding region of the factor VIII gene contains 70 CpG dinucleotides. In this study, we identified 9 different CpG hot spot mutations, of which 5 were not previously described. All told, a total of 25 of 70 CpG dinucleotides have been found to be altered by disease-producing mutations (refs. 6–8, 14, and 15; this work).

Complete characterization of mutations in a specific genetic disease may provide information about unsuspected and/or unrecognized regulatory elements of the gene responsible for the disease or may reveal the existence of an unknown gene(s) that also causes the phenotype of the specific disorder.

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