Molecular Defects in Hemophilia A: Identification and Characterization of Mutations in the Factor VIII Gene and Family Analysis

By Miyoko Higuchi, Lothar Kochhan, Rainer Schwaab, Hans Egli, Hans-Hermann Brackmann, Jürgen Horst, and Klaus Olek

Hemophilia A is an X-linked bleeding disorder caused by a deficiency or abnormality of factor VIII, affecting approximately 1 male in 10,000. A subgroup of the patients develops inhibitors against factor VIII during substitution therapy. Because a considerable percentage of all cases is thought to result from de novo mutations, it is likely that many different molecular lesions lead to hemophilia A. In order to understand the molecular basis of this disorder, we examined 160 patients with different clinical features using factor VIII gene probes. We could identify six different deletions and seven nonsense mutations within the factor VIII gene. Family analysis revealed that five of these mutations occurred de novo within two generations; two of them arose in the maternal grandfather and three in the mother. In one of these mothers we could identify a mitotic origin. Mapping of the deletions showed no deletion-prone region within the gene. Furthermore, we could not find any correlation between the particular gene defects and "inhibitor" phenotypes.

MATERIALS AND METHODS

Subjects

Two hundred two unselected hemophilia A patients from 160 pedigrees who are in treatment at the Hemophilia Center in Bonn have been investigated to identify mutations in the FVIII gene. The patients studied are divided into two groups on the basis of their clinical severity.9 One hundred fifty-seven patients (129 families) are affected with severe hemophilia A and 25 of them (24 families) produce antibodies against FVIII after substitution therapy (inhibitor-patient). Forty-five patients (31 families) are moderate-to-mild hemophiliacs and none of them is an inhibitor-patient. Relatives of patients in which mutations causing hemophilia A were detected were also examined to analyze the inheritance pattern of the abnormal gene and the polymorphic markers.

Restriction Endonuclease Analysis

DNA was isolated from whole blood leukocytes anticoagulated in EDTA as previously described.17 DNA (5 μg) was digested with the appropriate restriction enzymes and electrophoresed in 0.7% to 1.5% agarose gels. The DNA was then transferred to nitrocellulose membranes by Southern blotting.18 For routine mutation screening, DNA was digested with Taq1 and EcoRI or Msp1 and electrophoresed in 0.9% agarose gels. Hybridization protocols have been reported elsewhere.19 Radiolabeling was by nick translation using a commercial...
DNAProbess

The following probes were used in this study:

1. Three cDNA fragments for mutation screening: probe A, probe B-II (a 2.2 kb BamHI/EcoRI cDNA fragment from probe B spanning parts of exons 14 to 26 and exons 15 to 25), and probe C.

2. Two probes for segregation analysis of polymorphic markers: StI4 (locus DXS52)20,21 and F8A (locus F8).22

3. For characterization of mutations: (a) Three subcloned cDNA fragments from probe A, including probe A-I (a 366-bp SstI/HindIII fragment containing exons 4 to 13), probe A-II (a 642-bp HindIII fragment spanning exons 4 to 7), and probe A-III (a 797-bp HindIII/KpnI fragment comprising exons 8 to 12). (b) Two genomic fragments from λCM10.11, including fragment A (a 2.7 kb EcoRI fragment from the 5' end of intron 22) and fragment B (a 1.1 kb BamHI/EcoRI fragment from the 5' end of intron 25). (c) Two genomic fragments from pLk17.09, including fragment I (a 2.7 kb EcoRI fragment from the 5' end of intron 13) and fragment III (a 1.1 kb XbaI/BamHI fragment from the middle of intron 14). The three cDNA fragments were provided by Dr. J.J. Toole (Genetic Institute, Boston), while the probes F8A and StI4 were provided by Drs. R.M. Lawn (Genentech, San Francisco) and J.L. Mandel (Strasbourg, France), respectively. Two clones, λCM10.11 and pLk17.09, were isolated and characterized in our laboratory.23

Oligonucleotide Hybridization Analysis

DNA (7 to 10 μg) was digested with EcoRI or Rsal and electrophoresed in 1% agarose gels. The gels were dried and hybridized with radiolabeled oligonucleotides for three hours at 42°C.24 End-labeling of 19 mer synthetic oligonucleotides (Table 1) was performed using a commercial kit (Boehringer Mannheim). Posthybridization washes were carried out either in 1 x SSPE, 0.1% SDS at 5°C below calculated melting temperature (Tm) for five minutes, or in 3 mol/L TMA.CI at 55 to 57°C for 30 minutes.25 For rehybridization the gels were denatured in 0.5 mol/L NaOH, 1.5 mol/L NaCl and neutralized in 0.5 mol/L Tris.HCl, pH 7.0, 1.5 mol/L NaCl at room temperature.

Amplification and Direct Sequencing of Genomic DNA

Genomic DNA from patient 273 and a normal male control were amplified in vitro by the polymerase chain reaction (PCR) with Taq polymerase.26,27 Two oligonucleotides, 5'PCR-EX1718 from IVS-16 (5'-TGA TGA GAA GAA ATC CAC TCT GG-3') and 3'PCR-EX1718 from IVS-18 (5'-ACT GAT TGT TGT CCC AGT GC-3'), were used as the 5'-primer and 3'-primer, respectively. Thirty cycles of PCR were carried out, each cycle consisting of 30 seconds of denaturing at 94°C, 30 seconds of reannealing at 55°C, and two minutes of polymerization at 72°C using a DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT). The 778-bp PCR-product was purified on Microconcentrators (Centricon 30, Amicon, Danvers, MA) and sequenced directly using the method of Wong et al.28 The end-labeled oligonucleotide EX185P from the 3' end of exon 18 (5'-AG TGC CAT TTT ATA CTC CTC-3') was used as third primer.

RESULTS

Two hundred two unselected hemophilia A patients from 160 kindreds were studied by restriction endonuclease analysis using two enzymes, Taql and EcoRI or Mspl, and three cDNA fragments as hybridization probes. Thereby we could identify 13 molecular defects within the FVIII gene consisting of six deletions and seven Taql site alterations. Using our screening procedure described above, however, the vast majority of mutations were not detectable. All of the gene defects identified resulted in a severe hemophilia A associated with no detectable FVIII clotting activity. Six patients developed FVIII inhibitors (Table 2).

Deletion Mapping

A typical deletion mapping (patients 194/513) is shown in Fig 1.

Patients 194 and 513. Preliminary screening with probe B-II showed absence of the exon 14 fragments in patients 194 and 513. To localize these deletions a restriction analysis with several endonucleases was performed. Hybridization with fragment III (Fig 1A) suggested an identical deletion in both patients. Thus, we assumed them to have common ancestors. That was confirmed by several interviews with members of both families. The abnormal SstI and BamHI fragments are larger than the normal ones, indicating that they are junction fragments derived from two EcoRI fragments of 20 kb and 6.2 kb, and two SstI fragments of 20 kb and 7.4 kb, respectively (Fig 1A). That means that the 5' end lies within intron 13 and the 3' end within the 6.2 kb EcoRI fragment in intron 14. For precisely mapping the 3' end of the deletion, the PstI- and Mspl-digested DNA from patient 194 was hybridized with the same probe. This experiment revealed the 4.0 kb normal PstI fragment, but in place of the normal 4.4 kb Mspl fragment, an abnormal 5.2 kb fragment was found (data not shown). This observation proves that the 3' end of the deletion lies between the Mspl site and the PstI site on the normal 6.2 EcoRI fragment (Fig 1B). The 5' end point was determined by rehybridization with fragment I, which contains repetitive sequences. The observed PstI and EcoRI fragments had the normal size, but not the Mspl fragment. This finding demonstrated that the 5' end lies between the PstI and the Mspl site near the 5' end of intron 13 (Fig 1B). Precise localization of the 5' end was impossible, because single copy sequences were rare in this region. Consequently, the mutation of patients 194 and 513 represents a partial deletion of 12 to 16 kb including exon 14 (Fig 1B).

Patient 277. The deletion of patient 277 comprises the whole exon 26 or at least a large part of it, because no hybridizing signal corresponding to this exon is visible probing Taql-cleaved and EcoRI-cleaved DNA from the patient.

Table 1. Sequence of Oligonucleotides Used for Characterization of the Exon 22 and Exon 26 Mutations

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotide Designation</th>
<th>Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>22-N (normal)</td>
<td>5'CAG ACT TAT CGA GGA AAT T-3'</td>
</tr>
<tr>
<td></td>
<td>22-M (mutant)</td>
<td>5'CAG ACT TAT CGA GGA AAT T-3'</td>
</tr>
<tr>
<td>26</td>
<td>26-N (normal)</td>
<td>5'CGC TAC CTT CGA ATT CAC C-3'</td>
</tr>
<tr>
<td></td>
<td>26-M (mutant)</td>
<td>5'CGC TAC CTT CGA ATT CAC C-3'</td>
</tr>
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</table>
Table 2. Mutations In the FVIII Gene

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Severity of Hemophilia</th>
<th>Inhibitor Production</th>
<th>Mutation</th>
<th>Origin of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>194/513</td>
<td>Severe</td>
<td>+/+</td>
<td>Deletion 12-16 kb, exon 14</td>
<td>f</td>
</tr>
<tr>
<td>277</td>
<td>Severe</td>
<td>–</td>
<td>Deletion 1.8 kb, exon 26</td>
<td>f</td>
</tr>
<tr>
<td>484</td>
<td>Severe</td>
<td>–</td>
<td>Deletion 35 kb, exons 1-5</td>
<td>f</td>
</tr>
<tr>
<td>505</td>
<td>Severe</td>
<td>+</td>
<td>Deletion 15-20 kb, exons 7-9</td>
<td>Mother</td>
</tr>
<tr>
<td>580</td>
<td>Severe</td>
<td>–</td>
<td>Deletion 2.3-3.0 kb, exon 14</td>
<td>Grandfather</td>
</tr>
<tr>
<td>656</td>
<td>Severe</td>
<td>–</td>
<td>Deletion 1.7-2.0 kb, exon 3</td>
<td>Mother</td>
</tr>
<tr>
<td>273</td>
<td>Severe</td>
<td>+</td>
<td>Nonsense codon 1960, exon 18</td>
<td>f</td>
</tr>
<tr>
<td>450</td>
<td>Severe</td>
<td>–</td>
<td>Nonsense codon 2135, exon 22</td>
<td>Mother</td>
</tr>
<tr>
<td>509</td>
<td>Severe</td>
<td>–</td>
<td>Nonsense codon 2228, exon 24</td>
<td>Grandfather</td>
</tr>
<tr>
<td>418</td>
<td>Severe</td>
<td>+</td>
<td>Nonsense codon 2228, exon 24</td>
<td>ft</td>
</tr>
<tr>
<td>568</td>
<td>Severe</td>
<td>+</td>
<td>Nonsense codon 2326, exon 26</td>
<td>ft</td>
</tr>
<tr>
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<td>Severe</td>
<td>–</td>
<td>Nonsense codon 2326, exon 26</td>
<td>ft</td>
</tr>
<tr>
<td>693</td>
<td>Severe</td>
<td>+</td>
<td>Nonsense codon 2326, exon 26</td>
<td>ft</td>
</tr>
</tbody>
</table>

*This patient has been reported in reference 16.
†The proband’s mother carries the defective gene but without a positive family history.

Fig 1. Mapping of the exon 14 deletion. (A) Southern analysis of Kpnl-, SstI-, EcoRI- and BamHI-digested DNA hybridized with fragment III. The fragment sizes of the HindIII-digested DNA are shown on the right side of the autoradiograph (lane 1: patient 513, lane 2: patient 194, lanes 3 and 4: normal subjects). (B) Partial restriction map of the FVIII gene flanking the deletion. The hatched areas below the map represent the genomic probes (fragments I, II, III). Fragment II is identical to probe B-I. The filled triangles represent the absent restriction sites, the circles the present sites. The continuous lines show the gene region that is detectable with our probes. The hatched area below the map represents the minimum size of the deletion: Open bars indicate uncertainty about the full extent of the deletion.

with probe C. On the other hand, hybridization of the same filter with fragment B produced no abnormal pattern. That places the 5' breakpoint within intron 25, and the size of the deletion is at least 1.8 kb, including exon 26 (Fig 2A). A more accurate mapping was impossible, because we had no access to suitable genomic fragments.

Patient 484. Southern analysis of EcoRI-digested and SstI-digested DNA hybridized with probe A showed that several fragments corresponding to exons 1 to 6 are missing in the patient’s DNA. In order to determine the deletion end points, the same filter was probed to three subcloned fragments of probe A (A-I, A-II, A-III). The autoradiograph after hybridization with probe A-I showed no signal. The 5' end, therefore, is situated either within exon 1 or in the 5'-flanking region to the FVIII gene. Hybridization with a mixture of probes A-II and A-III revealed a new 8.0-kb EcoRI fragment replacing the normal 6.6-kb fragment of exon 6. No abnormal hybridization pattern was observed with probe A-II alone. The 3' end point maps to the normal 3.0 kb TaqI fragment within intron 9. The mutation of patient 484 is a partial gene deletion of at least 35 kb, including exons 1 to 5 (Fig 2A).

Patient 505. Hybridization of probe A to TaqI-digested DNA from patient 505 showed the absence of fragments corresponding to exons 7, 8 to 9, 10, and instead a new 1.75-kb fragment. Presence of the normal 6.4-kb SstI fragment and a new 8.8-kb EcoRI fragment replacing the normal 6.6-kb fragment indicate that the 5' end of the deletion lies between the SstI and the EcoRI site in intron 6. This abnormal 8.8-kb EcoRI fragment could be detected by both the probes A-II and A-III. On this account it represents a junction fragment derived from exons 6 and 10. Thus, the 3' end point maps to the normal 3.0 kb TaqI fragment within intron 9. The mutation of patient 505 is a partial gene deletion of about 15 to 20 kb extending exon 7 to 9 (Fig 2A).

Patient 580. The Kpnl- and BamHI-digested DNA from patient 580 was hybridized with probe B-I, a 2.5-kb EcoRI/BamHI fragment containing almost the entire exon 14. The normal 3.2-kb BamHI fragment was replaced by an abnormal 21-kb fragment, indicating the loss of the BamHI...
is present in the patient's DNA, because the absence of intron I 3 TaqI digestion. These results pointed to a point mutation in the FVIII gene as the defect, the short DNA segment flanking the altered TaqI site within exon 14 is consistent with these observations (Fig 2A).

Characterization of TaqI Site Alterations

Exon 18 mutation (Patient 273). In patient 273, an abnormal 5.0-kb TaqI fragment instead of the normal 2.2-kb fragment was observed when hybridization with probe B-II was performed. A normal pattern was seen after MspI digestion. These results pointed to a point mutation in the TaqI site of exon 18. In order to characterize the molecular defect, the short DNA segment flanking the altered TaqI site was amplified by polymerase chain reaction. Sequence analysis of the amplified DNA revealed a C to T substitution, which generates a nonsense codon (TGA) in place of the normal arginine codon (CGA) at position 2116 (Fig 2B).

Exon 22 mutations (Patients 450 and 509). In patients 450 and 509, an abnormal 12.8-kb TaqI fragment replacing the normal 5.8-kb fragment pointed to a C to T change in the TaqI site of exon 22. Hybridization of EcoRI-digested DNA with probe 22-N, an oligonucleotide specific to the normal allele (Table 1), generated a signal at 5.3 kb in normal subjects but not in the patients. Probe 22-M, an oligonucleotide with a TGA codon at position 2116 (Table 1), hybridized to the 5.3-kb fragment from the DNA of the patients but not to that of normal subjects. These results prove that the absent TaqI site in exon 22 is the result of a C to T substitution generating a nonsense codon at position 2135 (Fig 2B).

Exon 24 mutations (Patients 418 and 568). An alteration of the TaqI site within exon 24 in two patients (418 and 568) was identified by restriction analysis. A C to T transition in the TaqI site of exon 24 results in a loss of this recognition site, thereby generating a new HindIII site. On this assumption, the HindIII-cleaved DNA from these patients hybridized successively with the two genomic fragments A and B. Hybridization with fragment A showed an abnormal 7.0-kb fragment instead of the normal 21-kb fragment for both patients. The same filter hybridized with the fragment B shows a variant 13.5-kb fragment replacing the normal 21-kb fragment. That proves the presumed C to T transition within the 21-kb HindIII fragment. The mutation changed an arginine codon (CGA) into a stop codon (TGA) at position 2209 (Fig 2B).

Origin of Mutations

To determine the origin of the mutation, we performed linkage analysis of the defective FVIII gene and polymorphic
markers in the relatives (46 females and 13 males) of patients in whom the mutation causing hemophilia A had been identified. Relatives of patients 277 and 580 were not available for study. However, both of them had positive family history of hemophilia A. We could determine the origin of the mutations in five cases; three mutations occurred de novo in the mother (families 450, 505, and 656) and two in the grandfather (families 418 and 580) (Table 2). In family 656, the mother was a somatic mosaic for the abnormal FVIII gene.16

**DISCUSSION**

Thirteen molecular defects causing hemophilia A reported here represent independent mutational events. Furthermore, we could determine five de novo mutations within two generations. These and the previously reported findings8-15 exemplify the observed heterogeneity of hemophilia A at clinical and biochemical levels and also support strongly Haldane's hypothesis that a considerable fraction of the cases is the result of new mutations.8-10,13,14,16

In our study, two de novo mutations were point mutations. Whereas the de novo deletion in the maternal germ cell (family 505) might be explained by a meiotic recombination error, the small exon 14 deletion (family 580) has another cause.29,30 Because it originated from the maternal grandfather, it is most probably the consequence of an intrachromosomal event as described by Lehrman et al.31,32 In these cases, the inverted Alu sequences may play a significant role. Such an intrachromosomal rearrangement, however, could involve not only repetitive sequences but also partially homologous short segments in an inverted orientation.33 It is striking that the extension and the location of this deletion and the exon 14 deletion (family B) reported by Youssoufian et al34 are very similar. Moreover, these mutations are the only deletions within the FVIII gene published so far that occurred de novo in paternal germ cells. Although their breakpoints are clearly different, they may have a common genesis. Both the intrachromosomal and interchromosomal rearrangement seem to be a conceivable mechanism for our large exon 14 deletion (patients 194 and 513), because the 5' and the 3' end are located in a region with repetitive sequences (unpublished data).

In our study, 3.7% of the hemophilia A patients exhibit gross gene deletions. Taking into account the previously described mutations of this type, the deletions appear to cover the entire gene, indicating no region that is prone to deletions (Fig 1A). This situation is different from the Duchenne muscular dystrophy (DMD) gene: Approximately 50% of the DMD cases display gross gene deletions. Moreover, a major part of them is located in the proximal part of the gene.35 Thus, in contrast to the DMD gene, the FVIII gene has no peculiarities facilitating deletions. Nevertheless, it is noteworthy that several deletion breakpoints are clustered in the large introns; five in intron 25 (22.4 kb) and three in introns 13 (16 kb) and 22 (32.4 kb), respectively.

Several recurrent nonsense and missense mutations in exons 18, 22, and 24 of the FVIII gene13,14 have been reported, supporting the hypothesis that CpG dinucleotides represent mutational "hot spots" in higher animals. The increased mutation rate of this dinucleotide is thought to be due to spontaneous deamination of the methylcytosine.36 Nevertheless, this mechanism could not explain sufficiently such a fact that a significant proportion of C to T transitions in the FVIII gene (13/15 reported [references 7, 8, 11-13 and this study]) has occurred on the coding strands, because usually the C residues of both DNA strands are methylated. For this observed inequality in the mutation rate, replication characteristics might play a role for such mutational events as discussed by Wu and Maeda.38

It was assumed that in hemophilia A patients with inhibitors, the development of immune tolerance is prevented by deletions.7 That is not consistent with recent results: None of the deletion patients of Youssoufian et al37 were inhibitor-positive. Only two of our six deletion patients suffered from inhibitors. Furthermore, one of our inhibitor-negative patients (patient 484) displayed a partial gene deletion comprising most probably the translation initiation codon, thus preventing any expression of FVIII antigen. Originally, the situation in hemophilia B seemed to be different, because a majority of the inhibitor patients have had gene deletions.7,37-39 However, those findings were biased by the small case number. According to recent results, the inhibitor production is not associated with gross gene deletions in hemophilia B as well.40 The correlation between inhibitor formation and point mutations has not been discussed in detail so far. Principally, nonsense mutations generating a truncated FVIII molecule lacking certain epitopes should also lead to the "inhibitor" phenotypes. The observations in this study (Table 2) do not indicate a correlation between nonsense mutations and antibody production. It is noteworthy, however, that the identification rate of mutations in our study is higher in inhibitor-positive patients (28%, 7/25 examined) than in inhibitor-negative patients (4%, 7/177 examined), indicating that the gross gene alterations detected here are probably not sufficient for the inhibitor production but might be necessary.

In 8% of the examined mutant genes, we could identify the molecular defect causing the disease; that is undoubtedly the most reliable tool for carrier detection and prenatal diagnosis. In eight of the affected families, the hemophilic appears to be a sporadic case. For such families, applicability of restriction fragment length polymorphism (RFLP) analysis is limited. Indeed, the carrier state could be excluded only in 40.6% of female relatives (13/32 tested) in our study. The remaining potential carriers would need the conventional methods. However, the defective gene is now known, so we were able to define their carrier status; eight women were identified as noncarriers and nine as carriers, including the mother of patient 656, who is a somatic mosaic. The mothers of patients 450 and 505 appear to be noncarriers. However, the recurrence risk for them may be higher than that for the normal females, because not only our finding of the postzygotic mutation but also the observations of the possible germline mutations in DMD families41,42 may suggest that such mutational events are not as rare as one assumed previously. In a sporadic case of hemophilia in which the
mutation is known and the mother appears not to carry the mutation, prenatal diagnosis should be offered.

ACKNOWLEDGMENT

We are grateful to Dr S.E. Antonarakis, in whose laboratory we could perform the characterization of the exon 18 mutation. We thank Drs E. Weiss and A.-M. Frischaufl for providing the genomic libraries, and Drs R.M. Lawn, J.I. Toole, and J.L. Mandel for providing probes F8A, subcloned cDNA (probes A, B, and C) and StI4, respectively. We are indebted to C. Meisen for his help in isolating the genomic clone ACM10.11; J. Heilmann for photography; and the staff at the Hemophilia Center in Bonn for collecting the samples. We also thank all patients and their family members for their cooperation.

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