Molecular Characterization of Human Factor $X_{San\ Antonio}$


Enzymatic amplification technique was used to isolate all eight exons and sequences around the splice junctions, putative promoter, and polyadenylation sites of human factor $X$ DNA from a patient with factor $X$ deficiency. Two genetic changes in factor $X$ have been observed in this patient. The patient is most likely a compound heterozygote since there is only 14% activity associated with factor $X$. A point mutation that resulted in the substitution of cysteine (TGC) for arginine (CGC) at amino acid 366 was found in exon VIII of one allele of the factor $X$ gene. This mutation, which occurs in the catalytic domain, can affect the formation of a disulfide bridge and thus could result in a reduction in factor $X$ activity. Sequencing all the regions around the splice junctions revealed a second mutation: a deletion of one nucleotide (TCCT to TCT) in exon VII that would cause a frame shift at amino acid 272 followed by termination. We have also shown that the point mutation in exon VIII creates an ApaI restriction site and destroys the HinP1 site. Enzymatic DNA amplification followed by restriction digestion provides a quick, reliable, and sensitive method for carrier detection and antenatal diagnosis in affected kindreds. This is the first characterization of factor $X$ deficiency at the molecular level. We propose to name this mutation Factor $X_{San\ Antonio}$. We have performed enzymatic amplification$^{13}$ of the proband's DNA followed by DNA sequence analysis of the coding regions of the factor $X$ DNA to characterize the molecular basis of the inherited deficiency. Two mutations, a substitution of T for C, and a frame shift mutation have been detected. The first mutation results in an amino acid substitution in the catalytic region. The deduced amino acid substitution in the catalytic domain can affect the formation of a disulfide bond, possibly altering the activity of the protein. In addition, we have demonstrated a quick and reliable method for screening for the presence of the mutant allele; this should facilitate antenatal diagnosis and carrier detection in kindreds at risk for this defect.

MATERIALS AND METHODS

Peripheral blood samples were collected from a factor-$X$-deficient patient and the patient's mother; the father was unavailable for this study.

Factor $X$ activity was measured by a one-stage assay using factor-$X$-deficient plasma as a substrate. The prothrombin time (protime), activated partial thromboplastin time (APTT), and factor assays were performed as previously described.$^{14}$

The factor $X$ antigen levels were measured by crossed immuno-electrophoresis$^{15,16}$ methods and Western blotting.$^{17,18}$

The patient’s peripheral blood was used to prepare genomic DNA according to the method of Blin and Stafford.$^{19}$ This genomic DNA and that of a control subject was digested with EcoRI, BglII, and HindIII, and subjected to Southern blot analysis. The blot was probed with radio-labeled factor $X$ cDNA.

Characterization of the mutant factor $X$ gene involved isolating and sequencing all of the coding regions, splice junctions, putative polyadenylation signals, and promoter regions. Synthetic oligonucleotides derived from previously published intron sequences flanking the 5' and 3' ends of each exon were used for amplification of exons II through VIII.$^{19}$ The flanking sequences of exon I were obtained by constructing a partial genomic library of 20-kb BglII fragments in EMBL-3 (Stratagene, La Jolla, CA), plaque-purifying a clone hybridizing to an exon I probe, digesting the insert with HindIII and subcloning into pUC18; subclones hybridizing to the factor $X$ cDNA were sequenced and showed inserts containing exon 1 and the 5' and 3' flanking regions. HindIII restriction sites were built in at the 5' ends of each oligonucleotide except in the case of exon VIII, where two sets of oligonucleotides were used and only one set (H1,H2) contained HindIII restriction sites. Exons III and IV.

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ApaL22 identified the mutant allele was subcloned into the SmaI corresponding site in pUC I 8 vector; the other fragments were containing primers were cleaved with HindIII and subcloned into the addition, the amplified fragments generated by the HindIII site-and some were subjected to direct double stranded dideoxy sequencing. The possibility of error in copying the DNA using TaqI using Sequenase (US Biochemical Corp, Cleveland). In addition to the HindIII site-containing primers H 1 and H2, primers HA and HB (without HindIII sites) were used to amplify exon VIII. The target sequences were amplified in a 100 L volume containing 1 μg of genomic DNA in a mixture of 50 mmol/L KCl, 10 mmol/L Tris- HCl (pH 8.4), 1.5 mmol/L MgCl2 and oligonucleotides at 1 mM each dNTP (dATP, dCTP, dTTP, dGTP) at 200 μmol/L, and 2 units of TaqI underlined sequences were altered from the original to create HindIII sites for subcloning.

Table 1. Primers Used for Enzymatic Amplification for Exons I Through VIII and Their Locations in the Human Factor X Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Name</th>
<th>Sequence (5′ to 3′)</th>
<th>Distance From 5′ End of Primer to Corresponding Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>I A1*</td>
<td>GACAACAGCCATCCAAGCTTGCCGTGGAGAC†</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>I A2</td>
<td>CTGCGCCCCCTCCAAGCTTCGGGCTGCG</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>II B1</td>
<td>CAGGAAGCTTATGAAGGGAAGCTTGGT</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>II B2</td>
<td>ACAGAAGCTACGGGACGAGG</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>III C1</td>
<td>TCTAAGCTTTCTTATTAG</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>III D1</td>
<td>GTTATCTGAAGCTTAGGCGCAAGGTTAGCA</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>III D2</td>
<td>TCAAGAAGCTCCCGGGCAACTGACC</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>IV E1</td>
<td>CCGGCAAGCTTCCATCTCCAGCTTG</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>IV E2</td>
<td>ACAAGCTTGTGTCACCCACCCCTG</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>V F1</td>
<td>GCAGGTGGAACGTGAAGCTCTCTTGCTG</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>V F2</td>
<td>GAGGTGCAAGTGAACGTGAAGCTCTCTTGCTC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>VI G1</td>
<td>GGATGGAAGCTTACCCAGGAGAGGAC</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>VI G2</td>
<td>GGTCTTTGAGAAGCTTACAGTGAAGCTGTC</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>VII H1</td>
<td>AGCAACAGCTTGCGGAGAGCTATGCTCCC</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>VII H2</td>
<td>AAGGGCGGAACTCTGCTCCAGACAGA</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>VIII HA</td>
<td>CGTCGTCGCCAGGGACGGGACAC</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>VIII HB</td>
<td>GAGTGGAACGTTCACCTTTAAGAGA</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* A1 and A2, B1 and B2, ... , HA and HB denote sets of primers from the corresponding exons.
† The underlined sequences were altered from the original to create HindIII sites for subcloning.
‡ Primers C1 and D2 were used to amplify exons III and IV as a unit.
§ In addition to the HindIII site-containing primers H1 and H2, primers HA and HB (without HindIII sites) were used to amplify exon VIII.

The patient developed brisk postoperative bleeding after surgery. Initial coagulation studies were performed after multiple transfusions, and they disclosed a prolonged prothrombin time that did not correct with vitamin K but responded to plasma infusions. Follow-up studies were done 1 month after surgery, 3 weeks after the last plasma infusion. These studies showed a prothrombin time of 20.6 seconds (normal <14.5 seconds), an activated partial thromboplastin time of 33.2 seconds (normal 30 to 45 seconds), and a factor X activity of 14%. The patient's bleeding time, fibrinogen, and assays for factor II, V, and VII were within normal limits. The patient's mother was also evaluated and showed a protime of 15.3 seconds, an APTT of 33 seconds, and a factor X activity of 53%. Other second stage factor assays were normal.

The factor X antigen levels of this patient have been shown by crossed immuno-electrophoresis to be 36% of the normal. Western blot data revealed a single factor X band detectable by factor X antibodies, and factor X antigen levels comparable with those found using crossed immuno-electrophoretic methods. The mobilities of the patient factor X on Western blots are similar to the mobilities of normal factor X.

Initial Southern blot analysis using EcoR I, BglII and HindIII restriction enzymes revealed patterns identical to the normal DNA controls except that the patient DNA exhibited an EcoRI polymorphism identical to the one reported earlier33,34 (the patient's mother was unavailable for this study). These results indicated that no gross gene deletion or rearrangements were present in the patient's factor X gene.

Fragments containing exons I through VIII and a portion of intron on either side of each exon were isolated by enzymatic amplification of the genomic DNA from the factor-X-deficient patient. Nucleotide sequence of all exons, exon-intron junctions and the 5' and 3' flanking regions of the genes, were determined. Sequence analysis revealed two differences between the normal sequence (as determined by Leytus et al1 and Fung, and as established in our laboratory21 and factor Xsa Antonio; they occurred in exons VII (Fig 1) and VIII (Fig 2), the exons coding for the catalytic domain. In exon VII a single nucleotide (C) was found to be deleted at residue 838 (nucleotides in this report are numbered according to reference 3). A single base change from C
Fig 1. Autoradiograph showing the sequence of the region coding for exon VII of patient factor X (panel 1) and of normal factor X gene (panel 2). Markers point to bands corresponding to AGA (TCT in the opposite strand of DNA) in patient factor X and to AGGA (TCCT in the opposite strand of DNA) in the normal factor X, representing the deletion of one nucleotide.

Fig 2. Autoradiograph showing the sequence of the region coding for exon VIII of factor X,, (panel 1) and of normal factor X gene (panel 2). Markers point to bands corresponding to TGC (Cys) in factor X,, and to CGC (Arg) in the normal factor X; this change (from C to T) results in the amino acid substitution.

Fig 3. ApaL1 restriction digest of DNA fragment including exon VIII from normal factor X (lane A) and factor X,, (lane B), amplified with primers HA and HB. Sizes of the bands are noted by numbers 1, 2, and 3 corresponding to 625 bp, 382 bp, and 243 bp, respectively. Undigested DNA in the 625-bp region of lane B represents amplified DNA from the normal allele.
to T, resulting in the alteration of Arg to Cys, was found at residue 1121 in exon VIII. For each exon, several independent clones were sequenced; in some cases direct sequencing was also performed on the amplified DNA. Of the clones representing exons VII and VIII, approximately 50% yielded mutant sequences; the remainder displayed normal sequences.

The base substitution in exon VIII creates a new recognition site for the enzyme *Apa* I and destroys a *Hin* I site. *Apa* I recognizes and cleaves the sequence GTGCAC that is present at this region in the mutant but not in the normal factor X gene. To verify this site in the mutant factor X gene, 625 base pairs (bp) of DNA was obtained by enzymatic amplification of both the patient and control genomic DNA using oligonucleotides HA and HB. Subsequent digestion of the patient’s DNA with *Apa* I results in the pattern shown in lane B of Figure 3, with bands of 243 bp and 382 bp. Subjected to the same procedures, the normal DNA control revealed no evidence of enzymatic digestion (lane A).

**DISCUSSION**

Factor X deficiency was initially described by Graham and Barrow and given the name Stuart factor deficiency. Since then several factor X mutants have been detected; however, other than the recent attempts to detect restriction fragment length polymorphisms related to factor X deficiency, the molecular basis of inherited variants of factor X have not been characterized.

The active site of the molecule consists of three principal amino acids participating in catalysis: His-276, Asp-322, and Ser-419 (amino acids in this report are numbered according to reference 3). In addition, the catalytic region has three loops caused by the formation of disulfide bridges. The Arg-366 to Cys-366 mutation could affect the formation of a disulfide bridge or in some other way change the overall conformation of the catalytic region, resulting in reduced activity of factor X. The second mutation generates a frame shift followed by a translational termination, truncating the protein, and presumably resulting in a nonfunctional factor X. A band corresponding to nonfunctional factor X was not observed on Western blots; therefore, it is possible that the protein may have been degraded. Since factor X deficiency is an autosomal recessive disorder and this patient has two mutations, each one could account for one defective allele. The low factor X activity presented the likelihood that the patient is a compound heterozygote with two defective alleles. The allele having the frame shift mutation would result in a completely defective protein that we were unable to detect, whereas the Cys-substituted allele would produce a less catalytically active protein accounting for the overall lower factor X activity in the patient.

In the case of the Cys-substituted allele, the presence of a new restriction site combined with the enzymatic amplification technique provides a rapid and sensitive means for identifying the mutant allele. This method should prove useful in carrier detection and antenatal diagnosis in affected kindreds. We propose to call this mutant protein Factor X_San Antonio.

**REFERENCES**


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