Deficiency in the A-Subunit of Coagulation Factor XIII: Two Novel Point Mutations Demonstrate Different Effects on Transcript Levels

By Hanna Mikkola, Martti Syrjälä, Vesa Rasi, Elina Vahtera, Eija Hämäläinen, Leena Peltonen, and Aarno Palotie

Congenital deficiency in coagulation factor XIII is a rare autosomal recessive bleeding disorder. Although the defect was characterized over 30 years ago, little is known about the molecular basis of the disorder. Here, we show two novel point mutations in the gene of the A-subunit of factor XIII in the genetically isolated population of Finland. All eight factor XIII-deficient families identified in Finland were studied. The exons of the gene of A-subunit were amplified individually by polymerase chain reaction and subsequently sequenced by single-strand conformation polymorphism. Sequence analysis of the abnormally migrating fragments showed two point mutations resulting in an amino acid alteration. A C-to-T transition at Arg-661 in exon XIV created a premature stop codon. This mutation was detected in six of the eight families, thus being the major alteration causing FXIII deficiency in Finland. In two of the six families, the patients were compound heterozygotes with the Arg-661-Stop mutation in one allele and either a T-to-C point mutation in exon VI or a thus far uncharacterized mutation in the other allele. The T-to-C transition in exon VI resulted in a substitution of threonine for methionine 242. The transition was found in one family only, where it was in the heterozygote form combined with the Arg-661-Stop mutation. To evaluate the consequences of these mutations, steady-state FXIII mRNA levels were quantitated by solid-phase mini-sequencing. In addition to the termination of translation 70 amino acids before the initial stop codon, the Arg-661-Stop mutation causes a 10- to 30-fold reduction in FXIII mRNA levels. This is also likely to result in a low translation level in the truncated polypeptide. In contrast, Met-242-Thr mutation does not seem to affect the level of mRNA. Here, the absence of a functional and immunodetectable protein is probably caused by an altered conformation of the mutant polypeptide, resulting in early degradation of the defective protein.

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Congenital deficiency of factor XIII (FXIII) is a rare autosomal recessive coagulation disorder, that is characterized by a tendency for spontaneous bleeding and impaired wound healing. The symptoms consist typically of delayed hemorrhages after mild trauma, including spontaneous bruising, hemarthrosis, intramuscular and intracranial hemorrhages, and spontaneous miscarriages. Bleeding of the umbilical stump in a newborn baby is a sign associated particularly with FXIII deficiency.1,2 To avoid serious bleeding complications, most patients need lifelong substitution therapy with cryoprecipitate or purified FXIII concentrate.

FXIII deficiency is usually caused by a defect in the catalytic A-subunit of FXIII. Diagnosis is made by a clot solubility test in 5 mol/L urea and by verification of the absence of the antigen of FXIII A-subunit or corresponding enzyme activity in plasma.3,4 FXIII (fibrin stabilizing factor) is a transglutaminase that acts in the final stages of blood coagulation. It catalyzes the formation of covalent c-(γ-glutamyl)-lysyl cross-links between fibrin monomers, thus converting the loosely bound fibrin meshwork into a stable clot. FXIII also cross-links several other proteins such as α-2 antiplasmin, fibrinectin, and collagen.5 The tetrameric proenzyme consists of two catalytic A-subunits and two carrier protein B-subunits.6,7 The A-subunit is synthesized in megacaryocytes/platelets and in monococytes/macrophages, where it exists in a dimeric form.8,9 It is also found in high concentrations in the placenta and uterus, where it originates from tissue macrophages.10,11 The gene coding for the 731 amino acid A-subunit of FXIII has been localized on chromosome 6p24-25 and spans altogether over 160 kb. It contains 15 exons12 that code for a 4-kb mRNA.9 Very little is known about the genetic defects behind FXIII deficiency. So far, only three mutations have been reported in the gene of the A-subunit,13,14,20 and two mutations in the gene of the B-subunit of FXIII15 localized on chromosome 1q31-32.1,22,23

The worldwide frequency of FXIII deficiency has been estimated at 1 patient in 2 million.2 In Finland, the disease is unusually common; there are 12 affected cases in 8 families in the population of 5 million. This most probably represents a consequence of the founder effect. The country was populated by a relatively small number of settlers about 2,000 years ago and has remained genetically isolated for linguistic and geographic reasons.24 The isolation has led to the enrichment of certain defective genes, whereas some disorders common in other Caucasian populations are almost missing. This phenomenon has created a concept known as the Finnish disease heritage, which consists of about 30 recessive diseases occurring in Finland at substantially higher rates than elsewhere.25 Because of this unique population history, it is realistic to hypothesize that the FXIII deficiency in Finland is caused by one major mutation originating from a common ancestor.

Our aim was to characterize the molecular background of FXIII deficiency in Finland. In this study, we identified two novel point mutations in the FXIII A-subunit gene in Finnish patients. One of the mutations was found in six of the eight families, and the other mutation was found in one patient only in the heterozygote form. The prevalence of both mutations was studied in a representative population sample. To further analyze the consequences of the mutations, we determined the steady-state transcript levels of the mutant mRNA species using quantitative solid-phase mini-sequencing.
MATERIALS AND METHODS

Patients. Twelve patients and 20 unaffected family members from eight families were studied. The patients had been previously diagnosed on the basis of a clinical bleeding tendency, an abnormal urea clot lysis test, and an undetectable amount of FXIII A-subunit and a reduced amount of B-subunit or rocket electrophoresis (Table 1). The clinical characteristics of the patients were classified according to the questionnaire of the European Thrombosis Research Organization Working Party on FXIII.

Isolation of DNA and RNA. Peripheral blood leukocytes were isolated by lysing the red blood cells by ammonium chloride according to the methods described by Malinen et al. Total RNA was isolated by guanidium isothiocyanate extraction and cesium chloride gradient ultracentrifugation. The concentrations were measured by photometric absorbance.

Reverse transcriptase (RT) reaction. A total of 2 μg total RNA was reverse-transcribed in 20 μL reaction mixture containing 20 pmol of the primer of each region (FXIII primers 18 and 29 in Table 2, AGA primers as described by Ikonen et al), 20 nmol of the nucleotides dATP, dGTP, dCTP, and dTTP, 20 U RNAase (Promega, Madison, WI), and 16 U of avian myeloblastosis virus (AMV) RT (Promega) in its buffer. The reaction was performed by incubating the samples in the reaction mixture for 1 hour at 42°C.

Polymerase chain reaction (PCR)-amplification. A total of 100 ng genomic DNA or 5 μL of the RT reaction product were amplified according to the methods described by Saiki. For mutation analysis, the exons encoding for the A-subunit protein were subjected to 30 cycles of amplification in 200- to 300-bp fragments with the primers shown in Table 2. The reaction conditions were optimized for each individual region, but the standard conditions were denaturation for 1 minute at 94°C, annealing for 1 minute at 62°C, and extension for 1 minute at 72°C. Radioactive PCR for single-strand conformation polymorphism (SSCP) was performed by adding 2.5 μCi of α-32P dCTP or dGTP (Amersham, Aylesbury, UK) to each PCR reaction. The radioactive PCR products were analyzed by SSCP. The PCR products were diluted 1:5 with 5.1% sodium dodecyl sulfate and 10 mmol/L EDTA and mixed with an equal volume of stop solution (Sequenase kit; US Biochemicals, Cleveland, OH). The samples were heated at 95°C for 3 to 4 minutes, cooled briefly on ice, and loaded on a 5% polyacrylamide gel (60:1 acrylamide: bisacrylamide; BioRad, Richmond, CA). Electrophoresis was run overnight at room temperature at 8 to 12 W, depending on the size of the PCR products. During the electrophoresis, which is performed in renaturating conditions, the DNA fragments make conformations according to their base sequence. The migration in the electrical field depends on the size and conformation of the DNA fragment, so that aberrant migration is usually detected both in deletions/insertions and single base substitutions. Each sample was run in at least two different conditions by varying the concentrations of glycerol (between 0% to 10%) or the Tris-borate buffer (pH 8.3). The gel was dried at 80°C for 1 hour, and the bands were visualized by autoradiography at −70°C for 1 to 2 hours.

Sequence analysis. For sequencing the regions where mobility shifts were detected, PCR was performed using primers, one of which was biotinylated at its 5’ end. The PCR product was purified with streptavidin-coated microbeads (Fluoricon; Baxter, Mundehein, IL) as described earlier by Syvänen et al. A total of 25 μL of the PCR product was incubated with microbeads in TENT buffer (0.01% Tween 20, 1 mmol/L EDTA, 50 mmol/L NaCl, and 40 mmol/L Tris-HCl, pH 8.0 to 8.8) for 0.5 hours at room temperature. The sample was washed with TENT buffer, and the bound PCR product was

<p>| Table 1. Clinical Characterization of the Finnish FXIII-Deficient Patients |
|---|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/ Sex</th>
<th>Severity of the Symptoms</th>
<th>Spontaneous Bleedings</th>
<th>Other Symptoms</th>
<th>Treatment</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>All1</td>
<td>36/M</td>
<td>Severe</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All2</td>
<td>31/M</td>
<td>Severe</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All4</td>
<td>29/F</td>
<td>Severe</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All5</td>
<td>26/F</td>
<td>Mild</td>
<td>(x)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All6</td>
<td>18/F</td>
<td>Severe</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All7</td>
<td>55/F</td>
<td>Severe</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All8</td>
<td>33/M</td>
<td>Mild</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All9</td>
<td>28/F</td>
<td>Moderate</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All10</td>
<td>38/M</td>
<td>Moderate</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All11</td>
<td>34/F</td>
<td>Moderate</td>
<td>(x)</td>
<td>(x)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>All12</td>
<td>61/F</td>
<td>Moderate</td>
<td>(x)</td>
<td>(x)</td>
<td>x</td>
<td>x</td>
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</tbody>
</table>

The severity of the disease is based on the frequency and severity of the bleeding complications before regular prophylactic treatment. Abbreviations: (x), mild symptoms that occur only after trauma; ---, no operations have been made without prophylaxis.

* Monthly prophylaxis with cryoprecipitate.
denatured by incubation for 5 minutes in 50 mmol/L NaOH. The unbiotinylated strand was removed by washing the product twice with TENT buffer. The single-strand template was sequenced directly by the Sanger dye cycle termination method\textsuperscript{38} using a commercially available kit (Sequenase version; US Biochemicals) according to the manufacturer's protocol.

**Solid-phase minisequencing.** The samples from all the patients, family members, and healthy controls were screened for each mutation by solid-phase minisequencing, which unequivocally identifies the genotype. The principle of the method is described in Fig I and was performed essentially as earlier described by Syvänen et al.\textsuperscript{39} In brief, for the minisequencing reaction, the PCR was performed asymmetrically with one 0.2-μmol/L biotinylated primer and one 1-μmol/L unbiotinylated primer. A total of 10 μL of the PCR product and 40 μL phosphate-buffered saline/0.1% Tween in bovine serum albumin were added into streptavidin-coated microtiter wells (Streptavidin-coated combiplate; Labsystems, Helsinki, Finland). The samples were incubated at 37°C with gentle shaking for 1.5 hours and afterwards washed 5 times with TENT buffer. The unbiotinylated strand was removed by denaturing for 5 minutes with 56°C/NaOH and washing as above. A total of 50 μL of reaction mixture containing 20 pmol detection primer, 2 pmol specific \textsuperscript{3}H dNTP (Amersham, Aylesbury, UK), 0.5 U Taq DNA polymerase (Promega, Madison, WI), and PCR buffer was added to each well, and the samples were incubated at 50°C for 20 minutes and washed as above. The hybridized primer was released by denaturing with NaOH, and the radioactivity resulting from the incorporated \textsuperscript{3}H-labeled nucleotides was measured from each well in a liquid scintillation counter (Microbeta; Wallac, Turku, Finland). The genotype was defined by the ratio of incorporated mutant versus normal nucleotides (r value).

To evaluate the frequency of the identified point mutations in the general population, four DNA pools, each of which were collected from blood samples of about 150 blood donors, were screened. The screening method was magnetic-bead minisequencing that provides a larger surface area for capturing the biotinylated template, thus increasing the sensitivity of the assay, and thus making it very suitable for searching for rare alleles.\textsuperscript{40}

**Northern blot analysis.** A total of 20 μg of total RNA was electrophoresed through 0.8% formaldehyde agarose gel according to standard protocols and transferred to a Pall Biodyne nylon membrane (Pall BioSupport, East Hills, NY). The filters were hybridized to a 2.3-kb FXIII cDNA probe,\textsuperscript{41} kindly provided by Dr D.W. Chung, University of Washington, Seattle, WA), or a 281-bp PCR probe produced from exon XII (primers shown in Table 2). \(\gamma\)-Actin served as a control in the analysis.\textsuperscript{42}

**FXIII mRNA quantitation.** For more accurate quantitation of FXIII mRNA, 5 μL of the RT reaction product was subjected to PCR and subsequent solid-phase minisequencing (primers shown in Table 2). The quantitation was based on a titration curve achieved by PCR and solid-phase minisequencing using precisely known amounts (10\textsuperscript{4} to 10\textsuperscript{2} molecules) of cloned FXIII cDNA as a PCR template. From the logarithmic standard curve, the amount of FXIII mRNA initially present in 0.5 μg total RNA can be approximated and compared with the average level of four control individuals. The amount of FXIII mRNA was related both to the level of total RNA and to a reference mRNA in each sample. A lysosomal enzyme aspartylglucosaminidase (AGA) served as a reference mRNA. It is a housekeeping enzyme and, therefore, is expected to have very limited individual and tissue-specific variation in its expression. AGA mRNA was quantitated as described above with primers described earlier by Ikonen et al.\textsuperscript{39} and the ratio of FXIII mRNA versus AGA mRNA was compared with the average value of four control individuals.

To determine the relative levels of different alleles in compound heterozygotes, minisequencing was performed on the sites of the mutations, and the level of each allele was approximated from the standard curve after correcting the specific activity of the test nucleotides (dCTP, 61 to 64 Ci/mmol; dTTP, 94 to 120 Ci/mmol).

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### Table 2. Primer Sequences for FXIII A-Subunit Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer No.</th>
<th>Forward</th>
<th>Primer No.</th>
<th>Reverse</th>
<th>Length of the Product</th>
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<td>Genomic primers for SSCP analysis and sequencing</td>
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<tr>
<td>II</td>
<td>1.</td>
<td>5'GACCCTGGTAAGCTCAAAATGTCC 3'</td>
<td>2.</td>
<td>5'bioACCCAGAGTGTTGGAGGAG 3'</td>
<td>183 bp</td>
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<tr>
<td>III</td>
<td>3.</td>
<td>5'bioTTGATTATTTTCTCTCAACTTGGT 3'</td>
<td>4.</td>
<td>5'CAATGGCTATTTCCACCCTGG 3'</td>
<td>228 bp</td>
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<tr>
<td>IV</td>
<td>5.</td>
<td>5'bioTTGGTGAATACCACTTCAACAGG 3'</td>
<td>6.</td>
<td>5'CCCTCAGACAGGTGGAGGAG 3'</td>
<td>267 bp</td>
</tr>
<tr>
<td>V</td>
<td>7.</td>
<td>5'TTGAAGATGGCATGCCTGGAC 3'</td>
<td>8.</td>
<td>5'bioTATGAAGTGGTTGGAGGAG 3'</td>
<td>227 bp</td>
</tr>
<tr>
<td>VI</td>
<td>9.</td>
<td>5'TTGAACATCTTGTGGCCTGAC 3'</td>
<td>10.</td>
<td>5'bioGCAAATGGCAGTTGAAACAGG 3'</td>
<td>157 bp</td>
</tr>
<tr>
<td>VII</td>
<td>11.</td>
<td>5'CAAACTAAAGAAGAATCTCTC 3'</td>
<td>12.</td>
<td>5'bioATCCATGGCAGTTGAAACAGG 3'</td>
<td>284 bp</td>
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<tr>
<td>VIII</td>
<td>13.</td>
<td>5'bioGTCGCTAGATGTGGTGAAGTCTGG 3'</td>
<td>14.</td>
<td>5'CAACCTGAATCTCTGGTGGAC 3'</td>
<td>176 bp</td>
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<tr>
<td>IX</td>
<td>15.</td>
<td>5'CGCTCACTTCTGGTGGAC 3'</td>
<td>16.</td>
<td>5'bioAGATCGAAGTGGTTGGAGGAG 3'</td>
<td>222 bp</td>
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<tr>
<td>X</td>
<td>17.</td>
<td>5'ATCAGAACATTTGTGGCAAGAC 3'</td>
<td>18.</td>
<td>5'bioCTCTGCGAAACAAAAAAGGTGAC 3'</td>
<td>210 bp</td>
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<tr>
<td>XI</td>
<td>19.</td>
<td>5'GTCACAGAATCTCTCGTGTAC 3'</td>
<td>20.</td>
<td>5'bioAAAATGGCAGTTGACATCCTGGT 3'</td>
<td>263 bp</td>
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<tr>
<td>XII</td>
<td>21.</td>
<td>5'CAAGGGAAGGAAGAATCTCTGGC 3'</td>
<td>22.</td>
<td>5'AAAGGCCTGAGGTCAGTGTC 3'</td>
<td>281 bp</td>
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<tr>
<td>XII</td>
<td>23.</td>
<td>5'TCAAGAAGAAAGCGGCTGTTGAC 3'</td>
<td>24.</td>
<td>5'bioGCTTGGTCTCTGCTCAGG 3'</td>
<td>200 bp</td>
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<tr>
<td>XIV</td>
<td>25.</td>
<td>5'GTCGTGGGCACACTCTAGTGTAGT 3'</td>
<td>26.</td>
<td>5'bioTTGAGAAAAGACACAGACAGG 3'</td>
<td>213 bp</td>
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<td>XV</td>
<td>27.</td>
<td>5'bioGACCTCTCGGACACTTCTCCTG 3'</td>
<td>28.</td>
<td>5'TCACATGAAGGAGTGGTCTTTG 3'</td>
<td>192 bp</td>
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<table>
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<th>cDNA primers for RT-PCR</th>
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<tbody>
<tr>
<td>VI-X</td>
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<td>XIV-XV</td>
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</table>

### Minisequencing primers

<table>
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<th>Exon</th>
<th>Primer No.</th>
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<th>Test Nucleotides</th>
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<tr>
<td>II</td>
<td>30.</td>
<td>5'CCACAGTGAGGGCTCAGGCG 3'</td>
<td>Val-34-Leu</td>
</tr>
<tr>
<td>VI</td>
<td>31.</td>
<td>5'GGACACTGCTAGTGATGTA 3'</td>
<td>Met-242-Thr</td>
</tr>
<tr>
<td>XIV</td>
<td>32.</td>
<td>5'ATCCCTTAAGAAGAACCTGTG 3'</td>
<td>Arg-661-Stop</td>
</tr>
<tr>
<td>XIV</td>
<td>33.</td>
<td>5'ACAACTAGAAGATGTTCTCC 3'</td>
<td>Arg-681-His</td>
</tr>
</tbody>
</table>
Computer analysis of the mutant polypeptide with the GCG program package. The consequences of the mutations on the secondary structure of the protein were analyzed with the GCG database using the peptide structure program.

RESULTS

Screening for mutations by PCR-SSCP. To screen for mutations of the A-subunit gene of FXIII, the genomic DNA of one affected individual of each family was analyzed by PCR-SSCP. A set of 28 primers was designed to amplify individually all the exons coding for the amino acids. The PCR products of the patients were indistinguishable from the healthy controls in agarose gel, suggesting that no major deletion or insertion in the exon area was responsible for the disease. The conditions for SSCP analysis were optimized by limiting the size of the PCR products to about 200 to 300 bp and by varying the buffer and glycerol concentrations in electrophoresis. In SSCP analysis of the eight families, a total of three different mobility shifts was detected in the amplification products of exons II, VI, and XIV. In two of the families, no mobility difference between the patients and healthy control individuals was detected in SSCP.

Sequence analysis of the exons showing mobility shifts in SSCP. To pinpoint the nucleotide alterations causing the mobility shifts in SSCP, the abnormally migrating fragments were sequenced by the Sanger dideoxy chain termination method. Sequence analysis showed three different nucleotide alterations (Figs 2 and 3), all of which change an amino acid codon.

One of the mutations was a C-to-T transition in exon XIV changing the codon CGA to TGA. It causes the substitution of a premature stop codon for arginine in amino acid position 661 and results in the termination of translation 70 amino acids before the initial stop codon. Another point mutation was a T-to-C transition in exon VI changing the codon ATG to ACG and subsequently causing a substitution of threonine for methionine in position 242.

The third nucleotide alteration detected by sequencing the fragments migrating abnormally in SSCP was a G-to-T transition in exon II causing the substitution of Leu for Val-34.

No other mutations were detected by sequencing the coding regions.

Screening for the identified mutations by solid-phase minisequencing. To further confirm the nucleotide alterations and to show their cosegregation with the disease, the DNA samples of the patients and healthy family members were analyzed by the solid-phase minisequencing technique. The genotype was defined by the \( r \) value obtained from the counts of the mutated versus normal nucleotides. The \( r \) value ranged from 0 to 1 in the homozygotes, from 0.1 to 0.9 in the heterozygotes, and from 0.01 to 0.05 in the individuals who did not carry the particular mutation. Of all the eight families, six patients in four families were homozygous for the Arg-661-Stop mutation (Fig 4 and Table 1). Three patients in two families were compound heterozygotes with the Arg-661-Stop mutation in one allele. One of these patients carried the Met-242-Thr mutation, and two carried a thus far unidentified mutation in their other allele (Fig 4 and Table 1).
Fig 2. (A) Sequence analysis of exon XIV in patient no. AI2 shows a homozygous C-to-T point mutation resulting in the substitution of a premature stop codon for Arg-661. (B) Sequence analysis of exon VI in patient no. AII2 shows a heterozygous T-to-C transition resulting in the substitution of threonine for methionine 242. The patient is a compound heterozygote carrying the Arg-661-Stop mutation in the other allele.

Fig 3. The A-subunit protein of FXIII is shown. Activation of the plasma proenzyme occurs by the release of an aminoterminal activation peptide by a specific cleavage by thrombin at Arg-37 and by the binding of calcium that induces a conformational change dissociating the A- and B-subunits and unmasking the reactive thiol-group at Cys-314. Inactivation occurs by a specific cleavage by thrombin at Lys-513. The fibrin-binding site is located somewhere between the two thrombin cleavage sites. Finnish mutations and the mutations reported earlier by *Kamura et al., **Boord et al., and ***Standen et al. are positioned on the polypeptide.
Therefore, the stop mutation accounts for 62.5% of all the mutated alleles of FXIII-deficient patients in Finland. The Met-242-Thr mutation was only found in one of the families in which the affected individual was a compound heterozygote with the Arg-661-Stop mutation in the other allele. No mutation could be detected in two of the families. These patients carried neither of the mutations reported here nor any of the earlier reported mutations.

The G-to-T transition in exon II, resulting in the substitution of Leu for Val-34, was detected in the homozygote form in one patient (Fam.E) linked with both the Arg-661-Stop mutation and the Met-242-Thr mutation. In the same family, the G-to-T change was also detected in the homozygote form in the patient’s healthy offspring. Additionally, it was detected in the heterozygote form in another family carrying the Arg-661-Stop mutation (Fam.F).

**Determining the prevalence of the mutations by magnetic bead minisequencing.** To evaluate the frequency of the observed nucleotide alterations in the general population, the leucocyte pools of about 600 blood donors were screened. Four DNA pools, each collected from the samples of about 150 donors, were analyzed for the specific nucleotide alterations by magnetic-bead minisequencing. The sensitivity of the method is about 0.05%; therefore, it would easily detect even one heterozygote individual carrying the mutation from the pools of 150 individuals with wild-type alleles. The Val-34-Leu change was detected in 23% of the control individuals; this suggests that the Val-34-Leu change represents a common polymorphism in the Finnish population. Neither the Arg-661-Stop mutation nor the Met-242-Thr mutation were detected among the 600 controls analyzed. This was in agreement with the assumption, based on the number of affected families, that the carrier frequency of FXIII deficiency in Finland is approximately 1 of 400.

**Quantitation of FXIII mRNA by Northern blot and solid-phase minisequencing.** The RNA isolated from the buffy-coat fraction of peripheral blood cells was studied to evaluate the quality and quantity of the mutant FXIII mRNA species. In Northern blot analysis, the Arg-661-Stop mutant and the Met-242-Thr mutant mRNA species were equal in size to the normal 4-kb mRNA. However, the density of the hybridization signals showed a dramatic reduction in the level of the Arg-661-Stop mRNA, whereas the signal of the Met-242-Thr mutant mRNA was of a comparable density, as could be expected for one allele with normal transcript level (Fig 5). More accurate quantitation of the steady-state tran-
script levels of the mutant alleles was performed by RT-PCR and solid-phase minisequencing (Fig 1). This method provides the results in numerical values that allow for an objective analysis of the results and also facilitate an allele-specific quantitation of mRNA species. The interindividual variation in the level of the Arg-661-Stop mutant mRNA between four homozygotes was from 2% to 15% of the mean level of FXIII mRNA observed for control individuals. The initial results that showed the amount of FXIII mRNA in 0.5 μg of total RNA were also expressed versus the reference mRNA to correlate the amount of FXIII mRNA to intact cytoplasmic mRNA in general. Here, we used as a reference the mRNA coding for a lysosomal enzyme, AGA. The results calibrated with AGA were very similar to the corresponding results achieved by proportioning FXIII mRNA to total RNA. The calibrated FXIII mRNA levels of the Arg-661-Stop homozygotes varied from 3% to 10% of the corresponding mRNA level of control individuals (Fig 6).

The effect of the Arg-661-Stop mutation on the level of mRNA was further confirmed by analyzing the sample of a compound heterozygote patient no. E112 by using the minisequencing. The patient was found to express both the mutated Arg-661-Stop and Met-242-Thr mRNAs, but the steady-state transcript level of the allele with the Arg-661-Stop mutation was only about 1 of 30 of that of the allele with the Met-242-Thr mutation. The mRNA level of the Met-242-Thr allele was detected in amounts close to those that might be expected in one normal allele.

Analysis of the RNA of a patient with heterozygous Arg-661-Stop mutation in one allele and a thus far unidentified mutation in the other allele (patient no. FIII) showed FXIII mRNA levels of about 2% of normal in both alleles (Fig 6). The mRNA levels were also very low (1.0% to 1.7% of normal) in the patients in the two families where the mutation has not yet been identified.

Computer analysis of the mutant polypeptide. The consequences of the Met-242-Thr mutation were analyzed using computer-assisted analysis of the secondary structure of the protein. The program predicted that the methionine 242 change to threonine raises local hydrophilicity of the mutated polypeptide region (Kyle-Doolittle prediction) and converts the secondary structure from an α-helix into more of a β-pleated sheet-like conformation (Garnier-Osguthorpe-Robson prediction).

DISCUSSION

The bleeding disorder caused by deficiency in FXIII was characterized over 30 years ago. However, the defects on the DNA level remained unknown, until the reports by Kamura et al18 and Board et al,19 describing the first mutations in the gene of the FXIII A-subunit, were published in 1992.

Here, we describe two novel mutations, one of which is the major cause of the FXIII deficiency in the Finnish population. This Arg-661-Stop mutation was found in six of the eight families, accounting for two-thirds (63%) of the mutated alleles that result in FXIII deficiency in this population. In spite of this enrichment, consanguinity was known in only one of the families. This enrichment is most likely the result of the founder effect and the consequent genetic
isolation. There are other known examples of recessive diseases in Finland where most of the affected individuals carry identical mutations.\(^4,\text{6}\) This is of considerable advantage in the screening of carriers in such a population, but, because of the rarity of FXIII deficiency, carrier screening has relatively restricted practical implications.

This is the first report of a mutation in the FXIII A-subunit gene affecting mRNA levels. The Arg-661-Stop mutation results in a reduction in the steady-state transcript level of FXIII mRNA to about 3% to 10% of normal. Such a dramatic effect on mRNA resulting from a stop mutation has also been described in the case of some other genes. However, it is not known how universal the phenomenon is, although it seems to depend both on the gene and on the position of the premature stop codon within the gene. Generally, it has been suggested that the closer to the 5' end the termination codon is located, the more likely the mRNA level is to be reduced. Why a low mRNA phenotype should be caused by a stop mutation is not completely understood. However, it has been suggested that the rate of transcription is not affected, whereas the processing and transporting of nuclear RNA or the stability of cytoplasmic mRNA is (for review see Cooper).\(^6\)

An opposite example where a premature termination codon in the FXIII A-subunit gene does not affect the mRNA level was the mutation reported by Kamura et al.\(^8\) It was a dinucleotide deletion in the intron B-exon III boundary, resulting in frameshift and a premature stop codon shortly thereafter. From the evidence of these two examples, there must be factors other than the distance of the premature stop codon from the transcription initiation site that affect the level of mutant FXIII mRNA.

The low Arg-661-Stop mRNA level presumably results in a low translation level of the polypeptide, most likely contributing to the deleteriousness of the mutation. On the other hand, even a low amount of functional FXIII would be sufficient to maintain proper hemostasis in the patient.\(^7\) Although the premature termination of translation does not delete any of the regions known to be crucial for catalytic activity (eg, the thrombin activation site, catalytic Cys-314, the proposed Ca++ binding site\(^8\) and the fibrin binding site\(^9\) [Fig 3]), the missing 70 amino-acid part of the polypeptide might play a part in the correct folding of a mature protein. The altered tertiary structure of the mutant protein might result in the loss of catalytic ability or might lead to an early intracellular degradation of the protein.

The other mutation, a T-to-C transition in exon VI resulting in a substitution of threonine for methionine 242, was detected in only one patient in a heterozygote form. Therefore, it very likely represents a relatively new mutation with no enrichment in the Finnish population.

The Met-242-Thr mutation is situated in exon VI, which preceeds the exon coding for the catalytically active site. Computer-assisted analysis of the secondary structure of the protein predicts a conformational change of the mutated polypeptide region. Because of its location, this might have a direct effect on the catalytically active site of the enzyme protein. However, because no FXIII protein was detected in plasma in rocket electrophoresis, in spite of normal steady-state mRNA transcript levels, it is possible that the conformational change also destabilizes the mutant polypeptide, leading to a premature degradation of the defective protein.

The phenotype of the patients varies from symptoms comparable with severe hemophilia to a mild bleeding tendency that is limited to pronounced bruising and bleeding complications occurring only after trauma or during operations (Table 1). Generally, all the other Arg-661-Stop homozygotes, except patient no. CI12, have a severe bleeding tendency with continuous symptoms unless prophylaxis therapy is administered. The compound heterozygote patient no. EI12, who carries both the Arg-661-Stop mutation and the Met-242-Thr mutation, is also severely affected. It appears that the Finnish patients who carry a thus far uncharacterized mutation have a decidedly milder form of the disease with only occasional bleeding complications. Because the population of patients carrying each mutation is limited, it has thus far not been possible to conclude whether the phenotype is caused by a particular mutation type. However, because one of the Arg-661-Stop homozygous patients has a milder form of the disease and the severity of the bleeding tendency varies significantly even within a family (eg, patients no. FI11 and FI12), it appears that other genes also influence the resulting phenotype.

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