Molecular Analysis of Polish Patients With Factor VII Deficiency

By Arnaldo A. Arbini, Dinah Bodkin, Stanislaw Lopaciuk, and Kenneth A. Bauer

We analyzed the mutations in patients from 10 Polish kindreds with a bleeding diathesis due to factor VII deficiency. Patients from eight families had plasma levels of factor VII coagulant activity (VII:C) and factor VII antigen (VII:Ag) that were less than 4% of normal. The coding sequence of the factor VII gene was amplified from genomic DNA by polymerase chain reaction (PCR). Sequencing demonstrated a C to T transition at position 10798 resulting in Ala294Val, a G to A transition at 10976 resulting in Arg353Gln, and a single base pair deletion at 11125 to 11128 causing a frameshift mutation in the triplet encoding amino acid 404. Homozygosity for the three sequence alterations was confirmed with the restriction enzymes Avall and MspI and allele specific PCR, respectively. A homozygous patient from a ninth family with levels of VII:C and VII:Ag of 4% and 17%, respectively, had Ala294Val and the frameshift mutation, but not Arg353Gln.

Investigation of a homozygous patient from a tenth kindred with VII:C and VII:Ag of 11% and 47%, respectively, demonstrated Ala294Val and Arg353Gln, but not the frameshift mutation. Based on the above data, we conclude that the frameshift mutation in the codon for amino acid 404 is associated with marked reductions in VII:C, Arg353Gln can decrease plasma levels of factor VII in the presence of other mutations in the factor VII gene, and Ala294Val results in a dysfunctional factor VII molecule.

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**MOLECULAR ANALYSIS OF FACTOR VII DEFICIENCY**

<table>
<thead>
<tr>
<th>Patient</th>
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<th>Bleeding</th>
<th>VII:C (%) (rabbit/human)</th>
<th>VII:Ag (%)</th>
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<td>M/31</td>
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<tr>
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<td>75/73</td>
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**Table 1. Hemostatic Characteristics of Patients From 10 Polish Kindreds With Factor VII Deficiency**

VII:C and VII:Ag results are expressed as percentage of normal plasma pool. Rabbit and human refer to the type of thromboplastin used for the VII:C measurements.

human tissue factor (RecombiPlasTin; Ortho Diagnostic Systems, Inc, Raritan, NJ), and bovine brain thromboplastin (Thrombotest; Immuno AG, Vienna, Austria). Unless otherwise stated, VIII:C refers to levels obtained using rabbit thromboplastin. Plasma VII:C levels were determined with an enzyme-linked immunosorbent assay using a commercially available kit (American Bioproducts Co, Parsippany, NJ). A normal plasma pool was constructed by mixing equal volumes of plasma from greater than 30 control subjects. This population consisted of healthy laboratory and medical personnel between the ages of 20 and 50 years, who gave a negative history for bleeding, as well as thrombosis, and were not taking any medications at the time of sample collection.

**DNA isolation and in vitro amplification using polymerase chain reaction.** DNA was purified from leukocyte nuclei obtained from whole blood by standard techniques. Oligonucleotide containing intrinsic sequence(s) flanking exons 1a through 8 were synthesized on an Applied Biosystems 381A DNA Synthesizer (Foster City, CA). Polymerase chain reaction (PCR) amplifications were performed using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). For single-strand conformation polymorphism (SSCP) analysis, PCR products were generated in 20-μL reaction mixtures that initially contained 160 ng of genomic DNA, 0.4 U of Taq DNA polymerase (Perkin Elmer Cetus), oligonucleotide primers at a concentration of 0.5 μmol/L each, dNTPs at a concentration of 100 μmol/L each, 2 μCi of (α-32P)-dCTP (3,000 Ci/mmol, 10 Ci/μL; New England Nuclear, Billerica, MA), 1 to 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl, pH 8.3 (at 25°C), 50 mmol/L KCl, and 0.01 mg/mL of autoclaved gelatin. For subcloning, sequencing, or performing restriction enzyme analysis, PCR products were amplified in 50-μL reaction mixtures with the aforementioned reaction components excluding radiolabeled dCTP. Each sample was subjected to 30 cycles of denaturation (30 s at 95°C), annealing, and extension (30 s at 72°C). A 5-minute extension step in the first cycle, a 1-minute extension step in the next three cycles, and a 5-minute extension step in the last cycle were always included.

For amplifying the exons of the factor VII gene, the sequences of the sense and antisense oligonucleotide primers, the sizes of the product obtained, and the annealing temperatures of the PCR reaction, respectively, were as follows: 1A, 5'-ACAGGCGGCGCCGCACTGCA-3', 5'-ACCAAGTTATGAGAAAGACC-3', 195 bp, 50°C; 2, 5'-GCGCGGCCGCAAGGACGCTC-3', 5'-CCGCCAGGCGCTGGTGTCAC-3'; 288 bp, 70°C; 3, 5'-GCTTACCGGTGTT GGTC-3', 314 bp, 55°C; 4, 5'-AGTCTAGGGCAAGGGACTGGT-3', 314 bp, 55°C; 5, 5'-TGGGACTGTTTGTTCACTACA-3', 273 bp, 55°C; 7, 5'-CAGAGAAACAATGAGCAGCAAT-3', 5'-GATTGTCCCTTTGCT TCTGGTAGG-3', 470 bp, 55°C; 8, 5'-AATGGCAGCAAGCCATCC-3', 5'-CCCGAGACGTTGCGAGA-3', 732 bp, 54°C. For the SSCP analysis, 8 additional sets of internal primers were synthesized to obtain two overlapping PCR fragments encoding this domain of the factor VII molecule (8a, 8b). These products were made in secondary PCR reaction mixtures containing 0.02 pmol of the fragment encoding exon 8, which had been previously generated from genomic DNA and purified from low melt agarose. The sense and antisense oligonucleotides, PCR product sizes, and annealing temperatures, respectively, were as follows: 8a, 5'-AATGGCAGCAACG CAATCC-3', 5'-CCCGTACCGACCTCGGGC-3', 305 bp, 54°C; 8b, 5'-CGTCTAGGGCATGTGCCCC-3', 5'-CCCGAGACGTTGCGAGA-3', 414 bp, 64°C. All primer sets were annealed for 1 minute except exon 2, for which a 2-minute annealing cycle was used.

**SSCP analysis.** The method of SSCP analysis described by Orita et al3,2 was used to screen for unknown mutations in the factor VII gene with the following modifications. We ensured that the purity and quantity of the various fragments generated by PCR were adequate by electrophoresing an aliquot in a 1.5% (wt/vol) agarose gel containing ethidium bromide and visualizing it under UV transillumination. A small quantity (1 to 2 μL) of the PCR reaction mixture was mixed with 9 μL of a solution containing 95% (wt/vol) formamide, 10 mmol/L NaOH, 0.05% bromphenol blue, and 0.05% xylene cyanol. Immediately before electrophoresis, samples were heat-denatured at 95°C for 2 minutes and placed on an ice bath for 15 minutes. Three microliters were then loaded onto a 35 cm × 45 cm × 0.4 mm gel cast using Hydrolink-MDE Gel (AT Biochem, Malvern, PA) in 54 mmol/L Tris-borate, pH 8.3, and 1.2 mmol/L EDTA.20,2 Electrophoresis was performed at 6 to 8 W constant power at room temperature for 14 to 20 hours. The gels were then transferred onto Whatman 3M paper (Whatman International Ltd, Markstone, England), dried under vacuum at 70°C for 1 hour, and exposed to x-ray film (XAR-5; Kodak, Rochester, NY) at ~80°C for 36 hours using an intensifying screen.

**Cloning and sequencing of PCR fragments.** The amplified PCR fragments were gel-purified from low-melt agarose and ligated into PPTBlue T-vectors (Novagen, Madison, WI). The inserts were sequenced by the dye-oxy chain termination method44 on an Applied Biosystems 373A DNA Sequencer. All the mutations were confirmed in at least two independent clones. Sequence analyses were performed using the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc, Madison, WI) from the Molecular Biology Computer Research Resource (Boston University, Boston, MA).

**Restriction enzyme analysis.** Restriction enzyme digestion of PCR fragments was used to depict segregation patterns of mutations that were found by sequencing to introduce or abolish a restriction site. All restriction enzymes and DNA molecular weight markers were purchased from New England Biolabs (Beverly, MA). Approximately 200 ng of PCR product purified from low-melt agarose was digested with 5 U of enzyme in a final volume of 20 μL. Restriction fragments were extracted with phenol-chloroform, and 10-μL aliquots were electrophoresed in a 6% (wt/vol) polyacrylamide gel containing 1 mol/L urea. After completion of the electrophoretic procedure, gels were stained with ethidium bromide and photographed under UV transillumination. For fragments, in which restriction enzymes created products under 40 bp, the products were radio-
labeled with [α-32P] dCTP in the PCR reaction as described above. After completion of the electrophoretic procedure, these gels were fixed, dried, and autoradiographed for 24 hours at −80°C using an intensifying screen.

**Allele specific PCR.** Primer-directed allele specific amplification was used to detect mutations that could not be tracked using restriction enzymes. Fragments spanning a mutation site were amplified by PCR and used as templates for allele-specific PCR (ASPCR). Oligonucleotides of 14 bp containing the mutant or normal base at its 3‘ end were used with a common second primer at concentrations of 0.3 μmol/L. PCR reactions were performed in a total volume of 20 μL with 0.02 pmol template, 0.4 U Taq DNA polymerase, dNTPs at a concentration of 70 μmol/L each, and MgCl2 at 1.0 mmol/L. Optimal annealing temperatures for each set of primers were previously determined, and amplifications were carried out for 25 cycles. After completion of the PCR, 6 μL of the reaction mixture were electrophoresed in a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and photographed under UV transillumination.

**Informed consent.** Informed consent was obtained from the patients. The study was approved by the Human Studies Committee of Brockton-West Roxbury Department of Veterans Affairs Medical Center, Beth Israel Hospital (Boston, MA), and the Institute of Hematology and Blood Transfusion (Warsaw, Poland).

**RESULTS**

We have investigated the molecular defects underlying factor VII deficiency in patients from 10 kindreds of Polish ancestry (Table 1). One affected patient from each family was studied in detail. Patients A through H had moderate to severe bleeding diathesis and levels of plasma VII:C and VII:Ag that were less than 4% of normal. Patients I and J exhibited a milder hemorrhagic diathesis than the other patients, and had VII:C levels of 4% and 11% and VII:Ag levels of 17% and 47%, respectively. Similar VII:C measurements were obtained using rabbit brain thromboplastin or human tissue factor (Table 1) or bovine brain thromboplastin (data not shown). We were also able to investigate both parents of patients A, I, and J, and their results are also shown in Table 1. None of the 10 patients were known to be related, and a history of consanguinity was obtained only when large PCR fragments are used, and the coding sequence of exon 8 is almost 600 bp in length. Therefore, exon 8 was amplified by PCR after subdividing it into two overlapping pieces of 305 bp (8a) and 414 bp (8b). In all 10 patients, we identified electrophoretic migration patterns distinct from those of normal individuals in the second portion of exon 8 (8b), as well as exon 5. For exon 8b, patients A through H demonstrated the same electrophoretic pattern, whereas patients I and J had their own distinctive pattern (Fig 1). These findings in conjunction with the levels of VII:C and VII:Ag suggested that patients A through H had the same molecular defect, whereas patients I and J were different.

To determine the molecular abnormalities in exon 8, PCR fragments encoding the entire exon from patients H, I, and J were subcloned and sequenced (Table 2). The following alterations were identified in two independent clones from patient H: a C to T transition at position 10798 in the gene (GCC to GTT) resulting in Ala294Val; a G to A transition at position 10976 (CGG to CAG) resulting in Arg353Gln, and a single base deletion of a C between positions 11125 through 11128 (GCC CCA to GCC CAT). This deletion causes a frameshift mutation in the triplet encoding Pro404, and abolishes the TAG stop codon following amino acid 406. Two independent clones from patient I demonstrated the same abnormalities at positions 10798 and 11125 to 11128, but did not have the substitution at position 10976 resulting in Arg353Gln. Four independent clones from patient J showed sequence alterations resulting in Ala294Val and Arg353Gln, but he did not have the single bp deletion at position 11125 to 11128. Clones encoding exon 8 from patients A and B demonstrated sequences that were identical to patient H (Table 2).

For exon 5, we observed that the abnormally migrating bands on SSCP analysis in patients A through J were present in three normal subjects, who also displayed bands with the normal electrophoretic pattern (data not shown). This suggested the presence of a sequence polymorphism in homozygous form, which was confirmed by subcloning and sequencing PCR fragments containing this exon from patients H, I, and J. A C to T transition was observed at position 7880 in the gene, which is a neutral dimorphism in the codon for His115. Sequencing was then performed on subcloned PCR fragments containing the exons 1a, 2, 3 + 4, 5, 6, and 7 in patients H, I, and J, and no additional substitutions were detected.

We next examined if patients C through G had the same sequence alterations as patients A, B, and H, as was suggested by the SSCP analysis. To accomplish this, we used the fact that the C to T transition at position 10798 creates a new AvaiI restriction site, and the G to A transition at 10976 abolishes a MspI restriction site. Restriction digests of the PCR product containing the second portion of exon 8 (8b) demonstrated that patients A through G, as well as J, all had the Ala294Val (Fig 2, Table 2) and Arg353Gln substitutions (Fig 3). In patients H and J, homozygosity for these sequence alterations was proven by performing restriction enzyme digests with AvaiI (Fig 2) and MspI (Fig 3, Table 2) on parental DNA. The brother of patient H was heterozygous for the two sequence alterations. A similar series of restriction enzyme digests were carried out on PCR fragments of exon 8b obtained from patient I, her affected

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**Table 2. Coagulation Data and Genetic Alterations in the Factor VII Gene of Polish Patients With Factor VII Deficiency**

<table>
<thead>
<tr>
<th>Patient</th>
<th>VII:C (%)</th>
<th>VII:Ag (%)</th>
<th>Ala294Val</th>
<th>Arg353Gln</th>
<th>Frameshift</th>
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<tr>
<td>A-H</td>
<td>&lt;2</td>
<td>&lt;4</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>17</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>J</td>
<td>11</td>
<td>47</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
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</tbody>
</table>

VII:C and VII:Ag results are expressed as percentage of normal plasma pool. VII:C values refer to those obtained using rabbit thromboplastin. Presence or absence of the sequence alteration in each allele is denoted by + or − signs, respectively.
sister, and her parents. These experiments showed that the patient and her sister were homozygous for Ala294Val (Fig 2), but not Arg353Gln (Fig 3).

The single base-pair deletion between positions 11125 to 11128 did not result in an altered restriction enzyme recognition site. Therefore, we used ASPCR to demonstrate homozygosity for this mutation. To this end, we first generated a PCR fragment from genomic DNA containing all of exon 8. An aliquot of this reaction mixture was then used as template in a second PCR reaction. The sense primer 5'-CGCTTCTCATTGGTCAGCGGC and the antisense primer 5'-CTAGGGAAATGGGG were used to amplify a normal 395-bp product, while the former primer and the antisense primer 5'-CTAGGGAAATGGGC were used to separately amplify the mutant 394 bp product. In all our patients, except J, a fragment of the appropriate size was generated only with the primers designed for the mutant allele, but not with those for the normal allele (Fig 4). PCR products were produced with both primer sets in the parents of patients H and I, as well as the brother of patient H, which indicated that they were heterozygous for the deletion.

**DISCUSSION**

Our investigations of the molecular defects underlying factor VII deficiency in 10 Polish patients (A through J) from different kindreds have identified four alterations in the coding sequence of the gene. These include substitutions of Val for Ala and Gln for Arg at amino acids 294 and 353, respectively, of the gene product. A single base-pair deletion near the carboxy-terminus of factor VII produces a frameshift mutation that is predicted to add 28 additional amino acids to the protein. We also detected a neutral dimorphism in the codon for His115 in all of our patients.

Patients A through H were homozygous for Ala294Val,
Fig 4. ASPCR analysis showing the presence of a deletion of a single C between positions 11125 to 11128 of the factor VII gene. Completely blackened circles and squares in the pedigrees denote individuals who are homozygous for the deletion, while heterozygotes are partially blackened. PCR fragments of 658 bp spanning all of exon 8 were first generated from genomic DNA, and used as templates in the subsequent ASPCR reactions. The sense primer used to amplify the second portion of exon 8 (8b) was used with 14-mer primers containing the normal or the mutant sequence at its 3' ends. The expected size of the fragments amplified from the normal and mutant alleles were 395 and 394 bp, respectively. Amplification products were analyzed in 1.5% agarose gels containing 0.5 μg/mL ethidium bromide. The result in a normal individual (N) with each set of primers is shown in the last lane of each gel. There are normally three restriction sites resulting in four fragments of 186, 81, 80, and 67 bp. The G to A substitution at position 10976 abolishes the restriction site between the 186-bp and 67-bp fragments, resulting in a new fragment of 253 bp. In (A), restriction digests of patients A through G and J show homozygosity for Arg353Gln. In (B), completely blackened circles and squares in the pedigrees denote individuals who are homozygous for the sequence alteration, while heterozygotes are partially blackened. Patient H is homozygous for Arg353Gln, as is the father of patient J. The parents and brother of patient H, and the mother of patient J are heterozygous. Patient I and her family have the normal sequence at this restriction site. Note that the 81-bp and 80-bp fragments run very closely to one another. The DNA molecular weight markers are an Haelll digest of φX174.

Fig 3. Mspl restriction enzyme analysis showing the presence of Arg353Gln. PCR fragments of 414 bp were amplified from genomic DNA using the primers for the second portion of exon 8 (8b). After digestion with Mspl, the products were subjected to electrophoresis in a 6% polyacrylamide gels and stained with ethidium bromide. A restriction digest of a normal individual (N) is shown in the last lane of each gel. There are normally three restriction sites resulting in four fragments of 186, 81, 80, and 67 bp. The G to A substitution at position 10976 abolishes the restriction site between the 186-bp and 67-bp fragments, resulting in a new fragment of 253 bp. In (A), restriction digests of patients A through G and J show homozygosity for Arg353Gln. In (B), completely blackened circles and squares in the pedigrees denote individuals who are homozygous for the sequence alteration, while heterozygotes are partially blackened. Patient H is homozygous for Arg353Gln, as is the father of patient J. The parents and brother of patient H, and the mother of patient J are heterozygous. Patient I and her family have the normal sequence at this restriction site. Note that the 81-bp and 80-bp fragments run very closely to one another. The DNA molecular weight markers are an Haelll digest of φX174.
Arg353Gln, and the frameshift mutation, and all had a moderate to severe bleeding diathesis with levels of VII:C and VII:Ag that were generally less than 2%. While these individuals resided in different areas of Poland and are known to be related, it is highly likely that a founder effect is responsible for the high frequency of this genotype. Patient I and her sister were homozygous for Ala294Val and the frameshift mutation, but not Arg353Gln. These women were moderate bleeders with levels of VII:C and VII:Ag of 4% and 12% to 17%, respectively. It is tempting to speculate that they had a distant ancestor in common with the former group of patients. This would have required a transition within a CG dinucleotide, which are known mutation hot-spots, in the codon for amino acid 353 of a single ancestor of patient I. Patient J was homozygous for Ala294Val and Arg353Gln, but did not have the frameshift mutation. He had a moderate bleeding history with levels of VII:C and VII:Ag of 11% and 47%, respectively. Based on the above data, we conclude that the frameshift mutation in the codon for amino acid 404 is associated with marked reductions in VII:C. Arg353Gln can decrease plasma levels of factor VII in the presence of other mutations in the factor VII gene, and Ala294Val results in a dysfunctional factor VII molecule.

From a previous study seeking to identify sequence alterations associated with differences in VII:C levels, it is known that Arg353Gln is a polymorphism associated with a modest reduction in VII:C.30 It occurs with an allelic frequency of 0.1 in normal individuals in the United Kingdom and the United States.23,29 Individuals homozygous for the Arg353Gln allele have a significant reduction in their mean VII:C levels to 75% of normal, while subjects homozygous for this polymorphism have levels of approximately 50%. It is unclear whether these individuals have VII:C levels that are reduced relative to VII:Ag. Green et al.32 postulated that Arg353Gln induces an alteration in charge leading to a conformational change in the factor VII molecule, which affects intracellular processing, and reduces hepatic secretion or accelerates its clearance rate from the circulation.

It should be noted that patient J and his father were both homozygous for Arg353Gln and had VII:Ag levels of approximately 50%. Since this patient and his father are homozygous and heterozygous for Ala294Val, respectively, our data suggests that this substitution has little effect on plasma VII:Ag measurements.

To understand the effect that Ala294Val and Arg353Gln might have on factor VII function, we compared the amino acid sequences of factor VII with those of human factor IX,34 factor X,35 prothrombin,36 protein C,37 and chymotrypsinogen38 using the GCG software package. The aligned sequences were analyzed using a model for trypsin-like domains of bovine coagulation serine proteases proposed by Furie et al.39 This model is based on the known three dimensional structure of pancreatic serine proteases and sequence homologies between these enzymes and serine proteases involved in blood coagulation. It recognizes seven constant regions (CR) and six variable regions (VR) that are generally well preserved in chymotrypsin, trypsin, thrombin, factor Xa, and factor IXa.

According to the model for factor IX, Ala294 is one of eight amino acids in VR4. The predicted location of this region is on the surface of the molecule surrounding the active site where it may play an important role in substrate recognition.39,40 Evidence supporting this putative function can also be inferred from the fact that substitution of the factor VII residue homologous to Ala294 in factor IX, Ala320, by the polar amino acid Asp results in a complete loss of factor IX function.41 The patient with this mutation had levels of factor IX coagulant activity and factor IX antigen of less than 1% of normal and 90%, respectively. The substitution of Ala294 in factor VII with another nonpolar amino acid, such as Val, is a relatively conservative change, and might be expected to have a moderate effect on the function of the protein.

The Arg353 residue is located within a group of 10 residues with limited homology denoted as VR6. This region is also predicted to be on the surface of the molecule, but distant from the active site, suggesting that it may not be involved in the substrate recognition process. To date, substitutions affecting four different residues have been reported to occur in VR6 of factor IX, and all of these patients had concordant reductions in their plasma level of factor IX coagulant activity and factor IX antigen.42

The purification and biochemical characterization of mutant factor VII proteins containing Ala294Val and Arg353Gln from stably transfected cell lines will ultimately allow us to definitively address the functional importance of the various sequence abnormalities.

ACKNOWLEDGMENT

We thank Dr Yale Nemerson (Mt Sinai School of Medicine, New York, NY) for facilitating this collaborative study of Polish patients with factor VII deficiency.

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