Exclusion of the First EGF Domain of Factor VII by a Splice Site Mutation Causes Lethal Factor VII Deficiency

By John H. McVey, Emma J. Boswell, Osamu Takamiya, Gabriel Tamagnini, Victor Valente, Teresa Fidalgo, Mark Layton, and Edward G.D. Tuddenham

We have studied a family with homozygous lethal, blood coagulation factor VII (FVII) deficiency. To identify the mutation responsible for the deficiency, exons 2 to 8 and the intron-exon junctions of their FVII genes were amplified from peripheral white blood cell DNA by polymerase chain reaction and screened by single-strand conformational polymorphism analysis. The fragment showing aberrant mobility was cloned and sequenced. We detected a single point mutation, a homozygous G to A substitution at nucleotide position 6070, in the invariant GT dinucleotide at the 5' splice site of intron 4. Homozygosity was confirmed by loss of a site for the restriction endonuclease Mlu I. Analysis of the splicing pattern of ectopic transcripts in lymphocytes in the parents revealed that this mutation is associated with skipping of exon 4, which produces an mRNA encoding FVII with an in-frame deletion of the first epidermal growth factor-like domain (EGF 1). Transient transfection of COS-7 cells with an expression vector containing the ΔEGF 1 FVII cDNA shows that this mutant protein is not expressed. The identification of the molecular basis of the FVII deficiency in this family allowed mutation-specific prenatal diagnosis to be performed in a subsequent pregnancy. In this family complete FVII deficiency is associated with a severe bleeding diathesis but no developmental abnormalities, lending weight to the hypothesis that fetal FVII is not required for the putative angiogenic functions of tissue factor in humans.

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intraventricular clots, and cerebral edema. (E) IV.1 died at age 1 month with cerebral hemorrhage and hydrocephaly. (F) IV.2 died at age 12 days with hydrocephalus, intraventricular clots, and cerebral edema.

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Construction of expression vectors for wild-type (wt) and ΔEGF 1 FVII. A cDNA for wtFVII was provided by Professor E. Davie (University of Washington, Seattle, WA). The ΔEGF 1 FVII was generated from the wtFVII cDNA by a two-stage PCR.\(^{11}\) Oligonucleotide primer pairs 5'-CGAGCGGCGAGGAAGCTTGCA-A-3', 5'-TCAGCTGTTGTATCTGGTAA-3', 5'-TGGTTGATTTTTTCTTCTG-3', and 5'-GCTGTTGCTGGAATTCTTTA-CAGTCAAGGAGTGACAGCTGGA-3' were used to generate fragments of the FVII cDNA, spanning nucleotides 8-350 and 466-664 respectively, which overlap by 41 bp corresponding to the desired join of exons 3 to 5. These fragments were gel purified and used in a subsequent PCR with two further oligonucleotide primers 5'-TCTCCACATGTCTCAGGCCC-3' and 5'-TTTITTTCTAGAATGGGTATTTTCCACATGGA-3'. The PCR product was cloned and the nucleotide sequence verified by the dideoxy chain termination method using cycle sequencing kit (PE Applied Biosystems, Warrington, UK). The fragment was then subcloned into the wtFVII cDNA as a HindIII-XhoI fragment replacing nucleotides 29-639. Finally, the wtFVII and the ΔEGF 1 FVII cDNAs were cloned into the expression vector pcDNA3 (Invitrogen, Leek, The Netherlands).

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**Fig 1.** The pedigree of the family. The propositus is indicated by an arrow. \(^{1}\) IV.1 died at age 1 month with cerebral hemorrhage and hydrocephaly. \(^{1}\) IV.2 died at age 12 days with hydrocephalus, intraventricular clots, and cerebral edema.

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3'-TTCTCACATGTCTCAGGCCC-3' of the FVII cDNA, spanning nucleotides 8-350 and 466-664 respectively, which overlap by 41 bp corresponding to the desired join of exons 3 to 5. These fragments were gel purified and used in a subsequent PCR with two further oligonucleotide primers 5'-TCTCCACATGTCTCAGGCCC-3' and 5'-TTTITTTCTAGAATGGGTATTTTCCACATGGA-3'. The PCR product was cloned and the nucleotide sequence verified by the dideoxy chain termination method using cycle sequencing kit (PE Applied Biosystems, Warrington, UK). The fragment was then subcloned into the wtFVII cDNA as a HindIII-XhoI fragment replacing nucleotides 29-639. Finally, the wtFVII and the ΔEGF 1 FVII cDNAs were cloned into the expression vector pcDNA3 (Invitrogen, Leek, The Netherlands).

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**Cell culture and expression of FVII.** COS-7 cells (European Collection of Animal Cell Cultures Catalogue [ECACC] No. 87021302) were grown in Eagle’s minimum essential medium (MEM), 10% fetal calf serum, 5,000 IU/mL strep, and 5 µg/mL soluble vitamin K (menadione; Sigma, Poole, UK) in a 5% CO\(_2\) atmosphere. Cells were transfected by electroporation using the Gene Pulser apparatus (Bio-Rad, Hemel Hempstead, UK) set at 250 V, 250 µF. Plasmid (50 µg) was used to transfect 2 × 10\(^7\) cells. One microgram of a β-galactosidase expression vector pH110 (Pharmacia Biotech) containing the SV40 promoter was used as a cotransfectant to correct for variation in transfection efficiency. The electroperated cells were transferred to a 10-mL volume of MEM and grown for 24 hours. The cells were then washed twice with phosphate-buffered saline (PBS) and grown in 10 mL of protein-free medium (Hybrimax; Sigma), 5,000 IU/mL strep, and 5 µg/mL soluble vitamin K (menadione; Sigma) for a further 48 hours. The conditioned media were then collected.

**Detection of FVII secreted from COS-7 cells.** The 10-mL conditioned medium was reduced to 1 mL by centrifuging the solution through the filter of a Centricon-10 concentrator (10,000 kD cut-off) (Amicon, Stonehouse, UK). FVII antigen was assayed using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (Stago Asserachrom; Shield Diagnostics, Dundee, UK) and Western blot analysis using a rabbit polyclonal antibody. FVII:Ag levels were referenced to a normal pool plasma which was calibrated against the first international standard for factors II, VII, IX, and X (National Institute for Biological Standards and Control [NIBSC], South Mimms, UK). FVII:Ag could be measured in the range 1.04 to 104 mU/mL. Equal amounts of protein of each sample were electrophoresed on 10% precast NuPage gels (Novex, Frankfurt, Germany) with MOPS buffer (50 mMol/L Tris-HCl (pH 7.7), 3.5 mol/L sodium lantyl sulfate, 1 mMol/L EDTA) at constant voltage of 200 V according to the manufacturer’s instructions. The proteins were transferred from the gel to a nitrocellulose membrane by electrophoretic transfer. FVII on the nitrocellulose membrane was detected using the rabbit polyclonal anti-human FVII detection antibody in the ELISA kit (Stago Asserachrom; Shield Diagnostics). The rabbit polyclonal antibody was detected using a horseradish peroxidase conjugated goat anti-rabbit IgG antibody (BioRad) and ECL+ chemiluminescence detection reagents according to the manufacturer’s instructions (Amersham International, Little Chalfont, UK).

**Protein and β-galactosidase assay.** Cells were obtained by brief exposure to trypsin/EDTA. They were then washed in PBS, transferred to a 1.5-mL microfuge tube, and centrifuged at 5,000g for 5 minutes. The pellet was resuspended in 500 µL of 0.25 mol/L Tris HCl, pH 8.0, 0.543

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DNA cloning and sequencing. The amplified DNA fragments were gel purified on a 2% agarose gel and cloned at the EcoRI V site of the plasmid vector pBSK (Stratagene, Cambridge, UK). The inserts were sequenced by the dideoxy chain termination method using the dideoxy chain termination method using cycle sequencing kit (Pharmacia Biotech).
and the cells were disrupted by three freeze/thaw cycles. The debris was eliminated by centrifugation at 12,000 g for 5 minutes at 4°C. Soluble proteins in the cell lysate (supernatant) were quantified using a Coomassie blue protein staining assay (BioRad). β-galactosidase activity was assayed as previously described.12

RESULTS

Case history and coagulation studies. A baby boy was referred to the Paediatric Hospital in Coimbra with vomiting, prostration, and sudden onset twitching. He had been delivered 10 days previously by forceps, after an uneventful pregnancy; the Apgar score was 9/10/10. He had been discharged home on the third day after birth and had apparently been healthy until that morning.

His parents are consanguineous and both enjoy good health. However, a baby girl from a previous pregnancy had died at the age of 1 month with cerebral hemorrhage and hydrocephaly (Fig 1, IV-1).

Physical examination showed a prostrated baby boy, slightly pale with hypertonicity of the limbs, pausal respiration, and tense fontanelle. A computed axial tomography (CAT) scan showed hydrocephalus with compression of the IVth ventricle, numerous intraventricular clots, and marked cerebral edema. Coagulation studies revealed a prolonged prothrombin time, whereas the activated partial thromboplastin time was within the normal range. The procoagulant activities of the propositus were all within the normal range except for FVII activity, which was less than 1% (Table 1). A diagnosis of factor VII deficiency was made and fresh frozen plasma was administered.

Lumbar puncture performed under cover of fresh frozen plasma showed hemorrhagic fluid under tension. Emergency external drainage of the hydrocephalus was performed by removing 85 mL of hemorrhagic fluid, resulting in transient relief. A control CAT scan showed extensive bilateral cerebral hemorrhage and he died 44 hours after admission.

Identification of the mutation in the FVII gene. To identify the mutation responsible for the FVII deficiency in this family, exons 2 to 8 and the adjacent intronic sequences of the FVII genes of the propositus were amplified by PCR. SSCP analysis of PCR products revealed no difference in mobilities between the propositus and normal controls analyzed in parallel except with PCR fragments which spanned exons 3 and 4 (Fig 2). Exons 3 and 4 were amplified and analyzed as a single fragment. To determine the nucleotide substitution responsible for the altered electrophoretic mobility detected by SSCP analysis, the PCR-amplified DNA fragment was cloned and sequenced. A G to A point mutation was found at nucleotide position 6070 (Fig 3). This mutation is in the invariant GT dinucleotide at the 5' splice site of intron 4. All 10 clones sequenced corresponded to the mutant FVII sequence. The mutation abolishes a MluI restriction site by changing the sequence from ACGGT to ACGCA. PCR fragments of exons 3 and 4 from the propositus and his parents were digested with MluI and analyzed by agarose gel electrophoresis and Southern blot analysis with the human FVII cDNA (Fig 4). The propositus had only a 310-bp band and was therefore homozygous for the loss of the MluI site. His parents had both the 310- and 261-bp bands, indicating that they are heterozygous for the mutation.

Characterization of the splicing events associated with this mutation. FVII is synthesized in the liver, a relatively inaccessible tissue. However, total RNA isolated from lymphocytes has been shown to contain mRNA transcripts for genes not normally expressed in these cells. The analysis of these so-called

Table 1. Coagulation Data

<table>
<thead>
<tr>
<th></th>
<th>FVII:C (%)</th>
<th>FVII:Ag (%)</th>
<th>FIX (%)</th>
<th>FII (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.5</td>
<td>75</td>
<td>60</td>
<td>120</td>
<td>90</td>
</tr>
<tr>
<td>III.6</td>
<td>75</td>
<td>48</td>
<td>140</td>
<td>80</td>
</tr>
<tr>
<td>IV.2</td>
<td>&lt;1</td>
<td>ND</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>IV.3 (cord blood)</td>
<td>62</td>
<td>ND</td>
<td>70</td>
<td>65</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
The levels of secreted FVII:Ag in conditioned media by both ELISA and Western blot analysis. The relative efficiency of transfection was measured by cotransfecting with a β-galactosidase expression vector and measuring β-galactosidase activity in the transfected cell lysates. The values of FVII:Ag determined by the ELISA were corrected for transfection efficiency. FVII was secreted by COS-7 cells transfected with the wt cDNA (35 mU/mL); however, no detectable FVII was secreted by cells transfected with the ΔEGF 1 FVII cDNA even after fourfold concentration (i.e., <0.26 mU/mL). Western blot analysis showed that the detection antibody from the ELISA kit recognized predominantly the heavy chain of fully purified recombinant FVIIa. FVII was detectable in the conditioned media from cells transfected with the wt cDNA but not in the conditioned media from cells transfected with the ΔEGF 1 FVII cDNA (Fig 6).

Mutation-specific prenatal diagnosis. While this work was in progress, the mother became pregnant again and requested prenatal diagnosis. Chorionic villus material obtained from the fetus in the 10th week of pregnancy was shown by SSCP and MluI restriction analysis of PCR-amplified exons 3 and 4 to be homozygous for the wt FVII sequence (Fig 7). A baby boy was born after an uneventful pregnancy and was clinically normal with FVII activity in the cord blood sample of 62% (Table 1).

DISCUSSION

In this paper, we describe the further characterization of a novel mutation in a patient with homozygous lethal FVII deficiency which we reported in a preliminary form.13 This
mutation has recently been reported in an Italian family with severe but nonlethal FVII deficiency due to double heterozygosity for the mutation described above and FVII T359M. The mutation was identified by SSCP analysis of PCR-amplified DNA and sequencing cloned PCR products. The propositus was found to be homozygous for a G to A substitution in the invariant GT dinucleotide in the 5’ splice junction of intron 4. The homozygosity was confirmed by restriction endonuclease digestion. Splicing defects are not an uncommon cause of human genetic disease; between 8% and 15% of known single–base pair substitutions disrupt the normal splicing of an mRNA transcript. The vast majority of known mutations that affect splicing are single–base pair substitutions within 5’ and 3’ splice sites. Mutations within a 5’ or 3’ splice site usually reduce the amount of mature mRNA generated and/or activate alternative “cryptic” splice sites in the vicinity. The use of cryptic splice sites results in the production of mRNAs which either lack a portion of the coding sequence or instead contain additional intronic sequences. An alternative consequence of a splice site mutation could be for an exon to be no longer recognized as such, and as a result, to be excluded from the mature mRNA, a process called exon skipping. The analysis of the actual consequence of this mutation for FVII mRNA processing could not be carried out in cells which would normally express FVII because it is synthesized in the liver, which is inaccessible without invasive biopsy. The existence of extremely low background levels of correctly spliced mRNA transcripts of tissue-specific genes has been shown in supposedly “nonexpressing” cell types such as lymphocytes. These so-called ectopic transcripts can be analyzed by PCR after RT. Analysis of the splicing pattern of ectopic transcripts in lymphocytes isolated from the parents of the propositus revealed that this mutation is associated with skipping of exon 4. The skipping of exon 4 would produce an mRNA with an in-frame deletion of 113 nucleotides which encode 38 amino acid residues (G47-H84) comprising the entire first EGF domain of FVII.

One might predict that this mutant protein would fold correctly and be secreted from the cell. However, it seemed probable from the FVII antigen-activity measurements made on plasma samples obtained from the parents (Table 1) that they have little if any circulating EGF 1–deleted FVII. Transfection of COS-7 cells with expression constructs containing either wt or ΔEGF 1 FVII cDNAs confirmed that the mutant protein is not expressed in detectable amounts, less than 0.26 mU/mL or at least 135-fold less than wt, consistent with the observed phenotype.

Multiple lines of evidence suggest that the EGF domains of FVII are important in the TF/FVII interaction. Chimeric FIX/FVII molecules only bind to TF when FVII-EGF1-EGF2 modules are present. A monoclonal antibody whose epitope has been mapped to residues 51-88 of FVII (EGF 1) has been shown to inhibit FVII activation and its binding to TF. Analysis of the molecular defects responsible for FVII deficiency has identified two independent mutations at Arg 79 in the first EGF domain, FVII-R79Q and FVII-R79W. Surface plasmon resonance studies of the interaction between recombinant FVII-R79Q and TF have shown a decrease in its affinity for TF. The three-dimensional structure of the TF/FVIIa complex determined by x-ray crystallography shows EGF1 interacting with TF residues identified by mutational analysis as being responsible for FVIIa affinity. Finally, alanine scanning mutagenesis of FVII provides evidence that EGF1 tethers the enzyme to TF. Plausibly, an FVII molecule lacking EGF1 would be nonfunctional, or have severely reduced specific activity. However, the data presented in this work show that such a molecule is not expressed or expressed at extremely low levels.

The formation of the bimolecular complex of TF and FVII is thought to be the primary event in the initiation of blood coagulation in vivo. The mutation described in this paper results in severely reduced expression of a probably nonfunctional FVII molecule. Consequently, according to current coagulation theory, blood coagulation cannot be initiated—a situation incompatible with life. The fact that the homozygous affected
children in this family survived to term suggests that the first serious hemostatic challenge occurs at parturition. Furthermore, the fact that both children had no detectable developmental abnormalities supports the hypothesis that FVII is not required for normal fetal development, thus the embryonic lethal phenotype associated with disruption of TF expression is independent of fetal FVII expression. This conclusion is supported by the recent description of the targeted disruption of the mouse FVII gene. FVII<sup>−/−</sup> mice develop normally to term but die at/or shortly after birth due to abdominal and intracranial hemorrhaging. It can be hypothesized that normal in utero development in mice and humans lacking FVII is due to maternal rescue by transplacental transfer of small but sufficient amounts of FVII. However, there is no evidence for maternal-fetal transfer of FVII in the FVII<sup>−/−</sup> mouse embryos.

In conclusion, we have identified a mutation in the donor splice site of intron 4 of the gene encoding blood coagulation factor VII, which results in skipping of exon 4 which encodes the EGF 1 domain. This mutant protein is not expressed in vitro at detectable levels. Identification of the genetic basis of FVII deficiency in this family allowed mutation-specific prenatal diagnosis to be performed successfully in a subsequent pregnancy.

REFERENCES

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