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Detection of missense mutations by single-strand conformational polymorphism (SSCP) analysis in five dysfunctional variants of coagulation factor VII

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Five unrelated subjects with dysfunctional coagulation factor VII (FVII) were studied in order to identify missense mutations affecting function. Exons 2 to 8 and the intron-exon junctions of their FVII genes were amplified from peripheral white blood cell DNA by PCR and screened by SSCP analysis. DNA fragments showing aberrant mobility were sequenced. The following mutations were identified: in case 1 (FVII:C <1%, FVII:Ag 18%) a heterozygous A to G transition at nucleotide 8915 in exon 6 results in the amino acid substitution Lys-137 to Glu near the C-terminus of the FVIIa light chain; in case 2 (FVII:C 7%, FVII:Ag 47%) a heterozygous A to G transition at nucleotide 7834 in exon 5 results in the substitution of Gln-100 by Arg in the second EGF-like domain; in case 3 (FVII:C 20%, FVII:Ag 76%) a homozygous G to A transition at nucleotide position 6055 in exon 4 was detected resulting in substitution of Arg-79 by Gln in the first EGF-like domain; in case 5 (FVII:C 10%, FVII:Ag 52%) a heterozygous C to T transition at nucleotide position 6054 in exon 4 also results in the substitution of Arg79, but in this case it is replaced by Trp; case 4 (FVII:C <1%, FVII:Ag 100%) was homozygous for a previously reported mutation (G to A) at nucleotide position 10715 in exon 8, substituting Gln for Arg at position 304 in the protease domain. Cases 1, 2 and 5 evidently have additional undetected mutations.

INTRODUCTION

Factor VII (FVII) is a trace vitamin K-dependent plasma glycoprotein that circulates in blood as a single-chain zymogen composed of 406 amino acid residues (M_r 50,000) (1). Upon vascular injury and in the presence of calcium, FVII forms a one-to-one stiochiometric complex with its cell surface co-factor tissue factor (TF). Once complexed to TF, FVII is cleaved to its active form, factor VIIa (FVIIa) and rapidly converts zymogen factor X and factor IX to the active enzymes. The formation of an active complex between TF and FVIIa is widely thought to represent the primary stimulus for blood coagulation.

FVII zymogen is converted to its active form by proteolytic cleavage at a single site (Arg 152-Ile 153), resulting in a two chain molecule composed of an N-terminal light chain, linked by a single disulphide bridge to a C-terminal heavy chain. The light chain largely consists of an amino terminal γ-carboxyglutamic acid rich domain, followed by two epidermal growth factor (EGF)-like domains. The heavy chain consists of the serine protease catalytic domain. The FVII gene consists of nine exons; exons 1a and 1b encode the 5′ untranslated region and most of the pre-pro leader sequence whereas exons 2–8 encode the mature protein (2).

Hereditary FVII deficiency is a rare coagulopathy, with an estimated incidence of 1 in 500,000 and only about 150 cases have been reported. The clinical expression of FVII deficiency is variable, and despite its key role in coagulation initiation there often appears to be little correlation between residual FVII activity measured *in vitro* and haemorrhagic symptoms in cases of congenital deficiency: the reasons for this discrepancy are not understood (3,4). In the present study, we have sought missense mutations in the FVII genes of five unrelated subjects with dysfunctional factor VII variants not associated with bleeding symptoms, by PCR-SSCP analysis and DNA sequencing.

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RESULTS

Exons 2 to 8 and the intron-exon junctions of the FVII genes of five unrelated patients with dysfunctional FVII variants were amplified by PCR. The PCR products with the exception of exon 7 showed one distinct band of appropriate size on agarose gel electrophoresis, and no differences were detected between the subjects and normal controls. The PCR products from exon 7 showed one major DNA band and either one or two minor bands when analyzed by agarose gel electrophoresis. A polymorphic repeat at the 3' exon-intron junction of exon 7 has previously been described and may account for the minor bands observed (5,6). SSCP analysis of PCR products from exon 7, however, revealed no difference in mobilities between FVII variant samples and normal subjects analysed in parallel.

SSCP analysis identified only a single PCR product with aberrant mobility relative to the normal control in each of the FVII variants. In case 1, two extra bands with slower migration were observed for exon 6 (Fig. 1A). In case 2, a band with slower migration than the corresponding normal control was seen for exon 5 (Fig. 1B). Exons 3 and 4 were amplified and analyzed as a single product, and in cases 3 and 5 these products showed aberrant mobility. To further localize the mutation, the PCR products were digested with the restriction endonuclease *Dde* I which cuts within intron 3. Subsequent SSCP analysis showed the shifted fragment to be associated with exon 4 in both cases (Fig. 1C, E). Exon 8 was amplified as a single product, but being too large for SSCP analysis was digested with various restriction endonucleases prior to analysis. The primary PCR product was 640bp and the fragment sizes following digestion with the restriction endonucleases were: *Nar* I, 289 and 351bp; *Bst*X I, 299 and 341bp; and *Pst* I, 275 and 365bp. In case 4, two extra bands with slower migration than the normal control were observed following digestion with *Nar* I (Fig. 1D). However, no difference in migration was observed following digestion with either *Bst*X I or *Pst* I (data not shown).

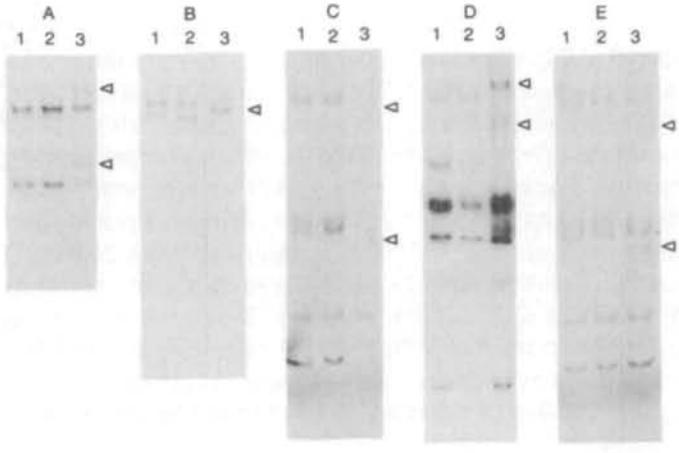


Figure 1. PCR-SSCP analysis in the FVII gene in five unrelated dysfunctional F VII variants. A: case 1, B: case 2, C: case 3, D: case 4 and E: case 5 show the results of PCR-SSCP analysis of exon 6, exon 5, exons 3 and 4, exon 8 and exons 3 and 4 including intron/exon junctions respectively. PCR-SSCP analysis of exons 3 and 4 followed digestion with *Dde* I digestion, and exon 8 after *Nar* I digestion. Arrows indicate extra bands with altered migration, when compared with normal control bands. 1 and 2: normal controls, 3: FVII variant.

To determine the nucleotide substitution responsible for the altered electrophoretic mobilities detected by SSCP analysis, each of the PCR-amplified DNA fragments was cloned and sequenced (Table 1).

Substitution of A by G at position 8915 was detected in exon 6 of case 1. As a result, the AAA codon for Lys-137 is replaced with the codon GAA for Glu. 9 of the 10 clones sequenced contained the mutant FVII sequence and 1 the wild type FVII sequence. Based on the SSCP analysis (Fig. 1A) this individual is most probably heterozygous for the mutation. The sequence was confirmed on both sense and antisense strands but no restriction enzyme site is created or destroyed to enable direct confirmation of heterozygosity.

In case 2, an A to G substitution was identified at nucleotide position 7834 in exon 5. The sequence of 5 of the 10 clones had the A to G substitution and the remainder corresponded to the wild type sequence. To confirm this mutation, the amplified DNA fragment including exon 5 and adjacent intron junctions was digested with the restriction endonuclease *Sca* I. The mutation abolishes a *Sca* I restriction site by changing the sequence from AGTACT to GGTACT. Three bands were observed corresponding to undigested DNA and the appropriate size fragments diagnostic for the presence of the *Sca* I site. It was concluded from the sequence and restriction analysis that this patient was heterozygous for the A to G transition at position 7834, which results in the substitution of Gln-100 by Arg.

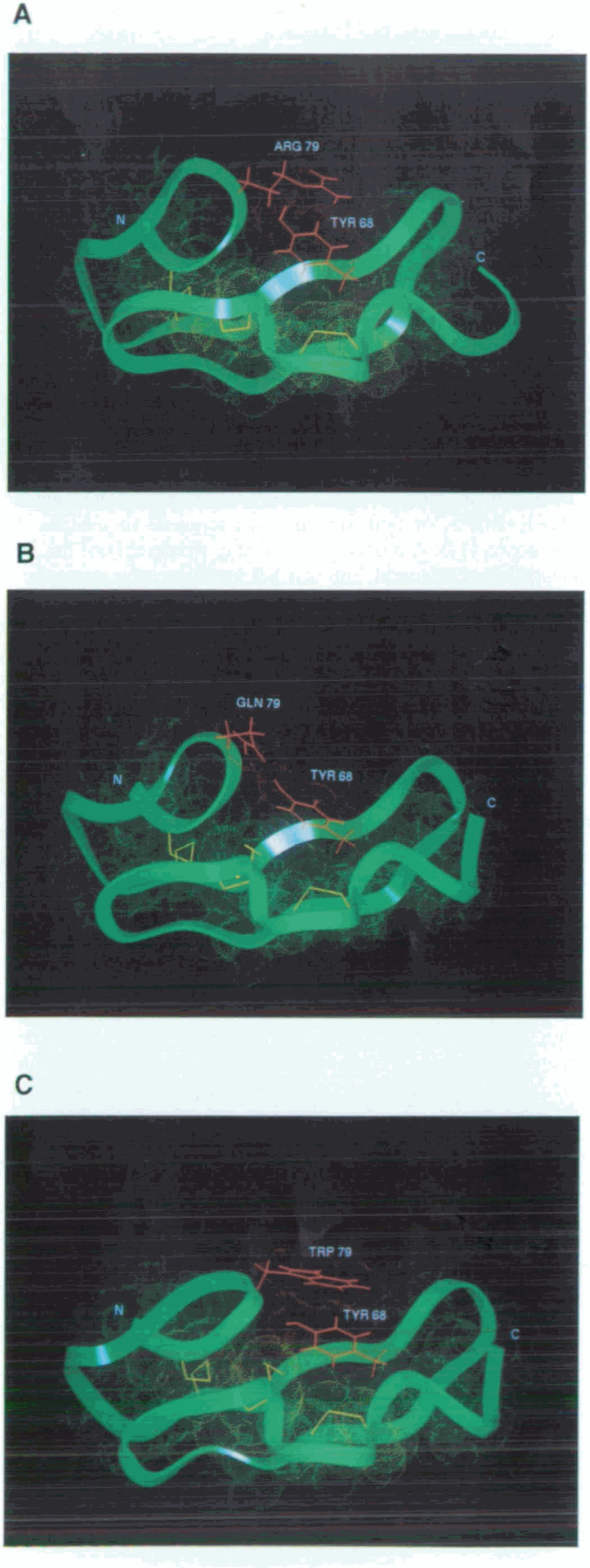
In case 3, a point mutation of G to A was found in exon 4 at nucleotide position 6055, resulting in the substitution of Arg-79 by Gln. All ten clones sequenced corresponded to the mutant FVII sequence. The homozygosity of this mutation was confirmed by loss of an *Msp* I restriction endonuclease site.

In case 4, transition of G to A was detected in exon 8 at nucleotide position 10715. This results in the substitution of Arg

Table 1. Phenotype and genotype data from five factor VII variants

Case No.	FVIIC* u.dl^{-1}	FVIIAg+ u.dl^{-1}	Exon	SSCP analysis§	Nucleotide position#	Base change	AA change	Restriction enzyme analysis§
1	<1	18	6	Hetero	8915	AAA to GAA	Lys-137→Glu	N.D.
2	7	47	5	?	7834	CAG to CGG	Gln-100→Arg	Hetero (*Sca* I)
3	20	76	4	Homo	6055	CGG to CAG	Arg-79→Gln	Homo (*Msp* I)
4	<1	100	8	Homo	10715	CGG to CAG	Arg-304→Gln	Homo (*Msp* I, *Pvu* II)
5	10	52	4	Hetero	6054	CGG to TGG	Arg-79→Trp	Hetero (*Msp* I, *Hae* III)

*Determined by one stage clotting assay using FVII deficient plasma and rabbit brain thromboplastin.
+Assayed using an ELISA kit (Diagnostica Stago,France) based on a polyclonal antibody to human FVII. A normal pooled plasma, assumed to contain 100u.dl^{-1} of FVII:C and FVII:Ag was used as control in both assays.
#Numbered according to O'Hara et al (2).
§Hetero = heterozygous mutant/wild type; Homo = homozygous mutation. N.D. = Not determined. ? = Indeterminate. Restriction endonucleases used to confirm the mutation are given in parentheses.



by Gln at position 304 in the catalytic domain of FVII. All ten clones displayed the mutant FVII sequence, strongly suggesting that this individual is homozygous for the mutation. This was confirmed by restriction endonuclease digestion, the mutation resulting in the creation of a *Pvu* II site and the loss of an *Msp* I site.

In case 5, a C to T transition was identified in exon 4 at nucleotide 6054, resulting in the substitution of Arg-79 by Trp. In this case, the individual was heterozygous for the mutation as determined by sequence analysis and by the heterozygous loss of a *Hae* III restriction endonuclease site.

DISCUSSION

Determination of mutations in dysfunctional FVII molecules provides important information to accompany protein modelling and structure-function studies. In this study, we have undertaken genetic analysis of five unrelated subjects who possess dysfunctional FVII variants. In all cases, mutations were detected in exons using PCR-SSCP analysis (Table 1). The mutations were then identified by sequencing cloned DNA and confirmed by restriction endonuclease digestion in 4 out of 5 cases. Three of the mutations detected have not previously been reported (cases 1, 2 and 5), one (case 3) has been described in a compound homozygote (7) and we have previously described the mutation in case 4 in an unrelated individual (8). The three mutations in codons 79 and 304 occur in CpG dinucleotides, a known hotspot for human gene mutation, and are either C→T or G→A transitions consistent with a model of methylation-mediated deamination of 5-methylcytosine (reviewed by Cooper & Krawczak (9))

The replacement of Lys-137 by Glu (case 1) in the connecting domain of FVII is a radical substitution, since the native lysine side chain is large and positively charged at physiological pH, whereas the glutamate group is smaller with a negative charge. This residue is adjacent to Cys-135 which forms a disulphide bridge with Cys-262 linking the light and heavy chains of FVIIa. It is difficult to predict the effect of such an amino acid substitution but the alteration of charge and shape and the resultant conformational change of this region of the molecule might interfere with chain folding and subsequent disulphide bond formation.

The mutation identified in case 2 results in the substitution of Arg for Gln-100 in the second EGF-like domain (EGF2). Gln-100 is highly conserved within the EGF2 domain of the vitamin K dependent serine protease family. In the fourth edition of the haemophilia B database (10), amongst 22 distinct missense mutations in EGF2 of FIX a missense mutation at the

Figure 2. Molecular models of the EGF1 domain of FVII based on the coordinates of the homologous domain in FIX (see text). The ribbon follows the polypeptide chain backbone, the amino (N) and carboxy (C) termini of the EGF domain are marked. Van der Waal's shells are displayed as dot surface and disulphide bonds are highlighted. **A:** The side chain of wild type Arg-79 projects horizontally into the solvent above the upper main cleft in the structure. **B:** Model of mutant detected in case 3, Gln-79, showing that its side chain projects almost vertically above the upper main cleft. **C:** Model of mutant detected in case 5, Trp-79, showing the aromatic side chain packed flat into the main cleft, against the side chain of Tyr-68.

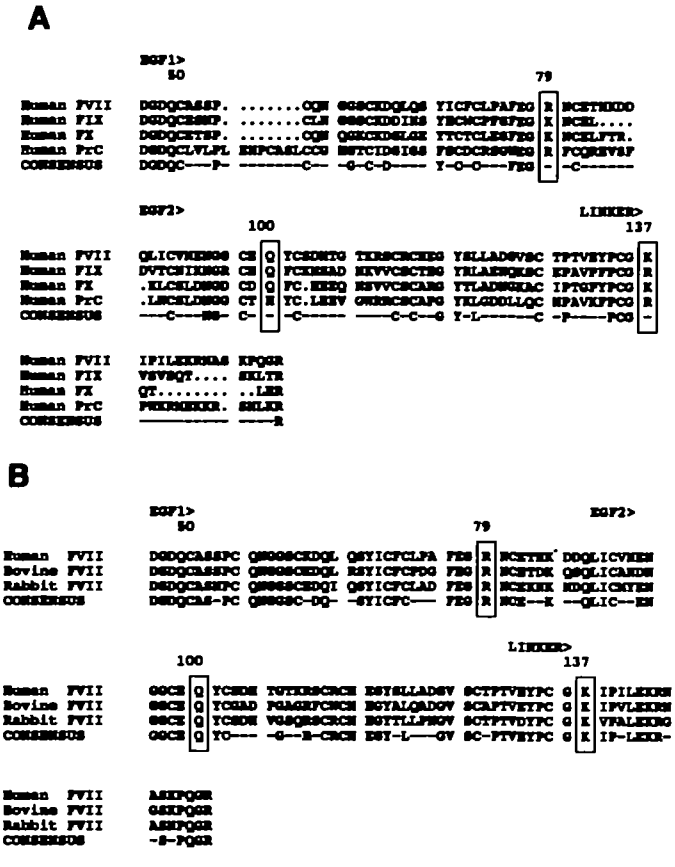


Figure 3. A. Comparison of the predicted amino acid sequences of human factors VII (17), IX (18), X (19) and protein C (PrC) (20) beginning at residue 46: EGF-like domains 1 and 2, plus the linking region to the carboxy terminus of the light chains are shown. B. Comparison of the predicted amino acid sequences of human (17), bovine (21) and rabbit (22) FVII. Sequence comparisons were displayed using the programs Lineup and Pretty (Genetics Computer Group, 1991). The sequences are numbered according to the mature human FVII sequence. The consensus sequence for each comparison is shown.

corresponding position (Gln-97 to Pro) has been described, but no phenotype data are available.

The mutations identified in cases 3 and 5 result in the substitution of Arg-79 by Gln or Trp respectively in the first EGF domain. Chaing and High have reported a FVII variant, FVII Charlotte, which was a compound homozygote, homozygous for both Arg-79 to Gln and for Arg-152 to Gln substitutions (7). It has been reported that the recombinant Arg-79 to Gln mutant does not bind its cofactor TF (11,12). In contrast, Kazama *et al* (13) have recently expressed and characterized recombinant FVII with the Arg-79 to Gln mutation and report that it exhibits TF-dependent activity indistiguishable from plasma derived FVII. Whilst these conflicting results require further investigation, our own data support a role for Arg-79 in factor VII structure or function, since we have identified two independent mutations at this residue associated with altered factor VII activity. We have modelled the EGF1 domain of FVII using the coordinates of the highly homologous EGF1 domain of FIX previously established by NMR spectroscopy. The side chain of Arg-79 does not form part of the integral structure of EGF1, but is in full solvent contact on the surface of the domain (Fig. 2A). The replacement of Arg-79 by Gln results in a change of shape and charge,

Table 2. PCR conditions used to amplify the human FVII gene

Exon	Denaturation	Annealing	Extension
2	96°C, 45 s		72°C, 135 s
3 and 4	95.5°C, 30 s	62°C, 15 s	72°C, 120 s
5	96°C, 30 s	65°C, 15 s	72°C, 120 s
6	96°C, 60 s	58°C, 60 s	72°C, 180 s
7	96°C, 45 s	60°C, 60 s	70°C, 128 s
8	96°C, 45 s	70°C, 60 s	70°C, 128 s

The PCR reaction was initially incubated at the denaturation temperature for each exon for 3 mins before undergoing 30 cycles of PCR using a PCH3 Techne cycler (Techne, Cambridge).

futhermore, the energy minimised model structure indicates a change in the orientation of the amino acid side chain (Fig 2B). Similarily, the substitution of Arg-79 by Trp results in a change in charge and the presentation of a bulky aromatic side chain on the surface of the domain (Fig. 2C). It has been suggested that the EGF domains of FVII are involved in determining its binding to its cofactor TF, however, it is not known whether EGF1, EGF2, or both domains are required to mediate this interaction (14). A monoclonal antibody whose epitope has been mapped to residues 51−88 of FVII has, however, been shown to inhibit FVII activation and its binding to TF, thus Arg-79 may be part of a binding site important in mediating the FVII/TF interaction (12).

The DNA sequences of other members of the family of vitamin K-dependent serine proteases, factor IX, factor X and protein C, have been determined. A comparison of the predicted amino acid sequence of EGF1, EGF 2 and the linker region between the light and heavy chains (Fig 3A) demonstrates that residues Arg-79, Gln-100 and Lys-137 are highly conserved. The equivalent residues in the 4 proteins are either identical or there is a conservative substitution. Furthermore, a comparison of the human with the rabbit and bovine FVII sequences demonstrates complete evolutionary conservation (Fig. 3B).

In conclusion, we have detected missense mutations in five independent dysfunctional human FVII variants, and we infer that these amino acid substitutions occur in regions of the molecule that are functionally important. Consistent with this view, each mutation results in radical changes of shape and charge in an amino acid side chain which is highly conserved both within the family of vitamin K-dependent serine proteases and between different species. *In vitro* expression and characterization of the mutant proteins are in progress in our laboratory in order to further define the functional effect of these mutations.

MATERIALS AND METHODS

Subjects

Five unrelated individuals from various countries were studied whose plasmas contain dysfunctional FVII as defined by reduced FVII activity in clotting tests using rabbit thromboplastin. None of these subjects has any significant bleeding tendency and all were detected after routine prothrombin time screening for reasons other than manifest haemorrhage. FVII activities and antigen levels of these subjects are shown in Table 1. Genomic DNA was prepared from peripheral blood leukocytes by established methods (15).

DNA amplification

Exons 2 to 8 of each subject's FVII genes were amplified by PCR using the oligonucleotides described previously (8). Five hundred nanograms of genomic DNA, 0.5μg of each oligonucleotide, and 1.5U *Taq* DNA polymerase (Promega) were added to 90μl buffer containing 1.5 mM $MgCl_2$, 10mM Tris-HCl (pH 8.3),

50mM KCl, 0.01% gelatin, 200μM each deoxynucleotide triphosphate and 2.5μCi of α-^{33}P-dATP (3000 Ci/mmol, 10 mCi/ml). The reaction mixture was then amplified using the conditions described in table 2.

SSCP analysis

SSCP analysis was performed according to the method of Hayashi *et al* (16). 3.5μl of the PCR product were diluted 10-fold in 85% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were then heated at 80°C for 3 mins to denature the DNA, snap-cooled on ice for 5 mins, before loading onto a 4.5% non-denaturing acrylamide gel (%T, 4.5%;%C, 2.25%). Electrophoresis was performed at 40W for 1.5 to 3 hours at 4°C. The gels were dried before autoradiography. Amplified products from exon 8 were digested with the restriction endonucleases *Nar* I, *Pst* I and *Bst*X I according to the manufacturers instructions, prior to SSCP analysis. Similarily, PCR products of exon 3+4 were digested with the restriction endonuclease *Dde* I digestion before SSCP analysis.

DNA cloning and sequencing

The amplified DNA fragments were gel purified on a 2% agarose gel and cloned at the *Eco*R V site of the plasmid vector pBSK (Stratagene, Cambridge, UK). The inserts were sequenced by the dideoxy chain termination method using a T7 DNA sequencing kit (Pharmacia, U.K.). Ten independent clones were sequenced for each fragment.

Molecular modelling

The first epidermal growth factor like domain of FVII was modelled on the homologous domain in FIX using atomic coordinates for the structure derived from nuclear magnetic resonance spectroscopy (kindly supplied by Prof. I.D.Campbell, Department of Biochemistry, Oxford). No insertions or deletions were necessary, therefore non-conserved residues could be replaced directly using the Biopolymer module of Insight II (Biosym Technology Inc., San Diego, Calif.) on a Silicon Graphics Personal Iris. The resulting structure was subjected to molecular dynamics simulation and energy minimised by the method of steepest descent. Residue substitutions were then made in this model corresponding to the Arg-79-Trp and Arg-79-Gln mutations described and the resulting structures finally subjected to further energy minimisation.

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