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Detection of two missense mutations and characterization of a repeat polymorphism in the factor VII gene (F7)

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Summary. The 3′ portion of the coagulation factor VII gene, containing the activation and serine protease domains, was investigated in four subjects with factor VII deficiency by temperature gradient gel electrophoresis and sequencing of polymerase chain reaction (PCR) products. Molecules displaying an altered melting behaviour were detected in three subjects, and direct sequencing showed two mutations. A G-to-T transversion causing a missense mutation, Cys-310 to Phe, suppresses a disulphide bond conserved in the catalytic domain of all serine proteases. This mutation, which in the homozygous form causes a severe reduction in protease activity (4%), was found in two patients from different Italian regions. A G-to-A transition, which gives rise to a missense mutation, Arg-304 to Gln, and is associated with the factor VII Padua variant, was found in the heterozygous form in a subject also affected by von Willebrand disease. Two polymorphic alleles, which differ in one repeat monomer element, were precisely mapped in a region spanning the exon-intron 7 border of the factor VII gene and studied in families with factor VII or X deficiency.

Introduction

Factor VII (F7, reviewed by Hedner and Davie 1989) is a vitamin K-dependent serine protease glycoprotein (Broze and Majerus 1980) involved in blood coagulation. It is synthesized in the liver and secreted as a single chain zymogen in the plasma, where it is activated ($FVII_a$) by the hydrolysis of a single peptide bond between Arg-152 and Ile-153 by thrombin, factor XII, X or XI (Radcliffe and Nemerson 1976; Kisiel et al. 1977; Seligsohn et al. 1979). It in turn activates factor IX or factor X in the presence of the tissue factor. The primary structure of F7 has been determined by cDNA sequencing (Hagen et al. 1986).

The gene, which is localized on chromosome 13q34–qter close to the factor X gene (Pfeiffer et al. 1982; de Grouchy et al. 1984; Gilgenkrantz et al. 1986) consists of nine exons and eight introns spanning 12.8 kb (O'Hara et al. 1987). Homology in the amino acid sequence and intron-exon organization with factor IX, X and protein C has been observed, thus suggesting a common origin by gene duplication (Long 1986; Leytus et al. 1986). The F7 gene contains five regions of tandem repeats (O'Hara et al. 1987; O'Hara and Grant 1988) in one of which we have recently reported a repeat polymorphism (Marchetti et al. 1991).

F7 deficiency (Alexander et al. 1951; reviewed by Hedner and Davie 1989) is inherited as an autosomal recessive disorder with high penetrance and variable expressivity (Triplett et al. 1985). Cerebral haemorrhage has been reported to occur in 16% of homozygous patients and thromboembolism has also been described (reviewed in Hedner and Davie 1989). High F7 activity is associated with risk of thrombosis and myocardial infarction.

F7 variants have been described (Girolami et al. 1977, 1979) and a few gene mutations have recently been reported (Chaing and High 1991; O'Brien et al. 1991).

The molecular characterization of F7 gene lesions and the study of their inheritance could improve the understanding of the F7 deficiency heterogeneity. Moreover these studies provide information for comparative structure-function studies (Kumar et al. 1991; James et al. 1991). Plasma F7 and its macromolecular complex could be a target for antithrombotic molecules and F7 has been produced by recombinant DNA techniques for the treatment of haemophilia (Thim et al. 1988; Hedner and Kisiel 1983; Rao and Rapaport 1990).

For the molecular characterization of mutations and polymorphism we amplified F7 gene regions coding for the activation and catalytic domains and containing repeated sequences. For a rapid study a modification of the denaturing gel technique (Fischer and Lerman 1983)

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coupled to direct sequencing of polymerase chain reaction (PCR) products was used.

Materials and methods

Patients

Five patients, four with F7 deficiency and one with factor X deficiency, and their families were studied. They were from different Italian regions (Emilia-Romagna, Veneto and Marche). R.D. (FVII:C 7%) had a prolonged bleeding time and frequent bleeding episodes, and had received several blood transfusions. S.M. (FVII:C 4%) had a sligtly prolonged bleeding time and a mild bleeding tendency. Her parents were heterozygous for Tay Sachs gangliosidosis. R.P. (FVII:C 12%) had a normal bleeding time and had had no bleeding episodes, not even after tooth extractions. L.M. is a 9-year-old child with a life-long history of bleeding. He had been complaining of epistaxis and gingival bleeding. His mother bled after tonsillectomy and tooth extractions. The father and the two sisters were asymptomatic. The patient came, originally, from the isolated area of north-eastern Italy (Piave river valley) where F7 Padua was first described and found to be widespread among the local population (Girolami et al. 1982). K.F. is a patient with classic factor X deficiency, affected by epistaxis, haematomas, haematuria and occasional haemarthrosis. This patient and his family have been reported previously (Girolami et al. 1975).

Activity and antigen

Samples were collected in sodium citrate (3.8%) as anticoagulant (1:9, v/v). F7 activity (FVII:C) was measured as previously described (Girolami et al. 1982). The substrate was a mixture of equal parts of F7-deficient plasma and adsorbed normal plasma. Rabbit brain thromboplastin (Dade Laboratories) and ox brain thromboplastin (Stago Laboratories) were used as tissue thromboplastin. F7 antigen (FVII:Ag) was evaluated by ELISA using the reagent kindly supplied by Stago Laboratories. Von Willebrand factor antigen was measured by ELISA. Von Willebrand factor ristocetin cofactor activity (vWF:RCoF) was determined as previously described (Bernardi et al. 1990).

Southern analysis

DNA from patients and controls was digested with restriction enzymes (Boehringer, New England Biolabs), Southern blotted, hybridized and washed as previously described (Bernardi et al. 1990). PCR fragment of (Fig. 1) was used as probe, after cloning in the TA cloning vector pCR1000 (Invitrogen).

PCR amplification

From the sequence of O'Hara et al. (1987) eight primers were synthesized: (a–h, Fig. 1). Primers a, c, e and g were from the coding strand: a, (GCATCTTTCTGACTTTTGTT); c, (AATGTGACTTCCACACCTCC); e, (GAGGTGGCAGGTGGTGGAAA); g, (CCACCTGCACGTGATCTGC). Primers b, d, f and h were form the complementary strand: b, (TAGACCCTCAGTGAGTGTC); d, GATGTCTGTCTGTCTGTGGA); f, (CGGCACAGAACATGTACTCC); h, (TGCATGGTGATGCTTCTGAA). Thirty PCR amplification cycles (Saiki et al. 1985; Innis et al. 1988) were run in a volume of 25 µl containing 1.6 units *Taq* polymerase, 0.1 µg human DNA, 7 pmol of each primer in 200 µ*M* dNTPs, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$. ab and gh fragments were obtained at: 92°C for 20 s, 56°C for 3 s and 70°C for 40 s; cd and ef at 92°C for 20 s, 57°C for 3 s and 70°C for 40 s; ad and eh at 92°C for 20 s, 56°C for 3 s and 70°C for 1 min 43 s (plus 1 s increase every cycle).

Temperature gradient gel electrophoresis (TGGE)

Crude PCR products, after denaturation and renaturation to favour heteroduplex formation, were analysed on an 8% polyacrylamide gel containing 4 *M* urea and superimposed on a linear temperature gradient produced on a plate (Diagen, Düsseldorf, FRG). Samples were run as described in the operation manual (TGGE system, Diagen) with minor modifications. PCR products were run under two different sets of conditions: temperature gradients perpendicular (T1 = 15°C, T2 = 61°C) or parallel (T1 = 29°C, T2 = 65°C) to the electrical field (500 and 300 V). Gels were fixed and silver stained.

Sequencing

The amplified fragments were excised from the gel, phenol extracted and sequenced (Sanger et al. 1977) with Sequenase (US Biochemical) after labelling with α^{35}SdATP. All sequences were determined at least twice. DNA and protein sequences were computer analysed using HIBIO DNASIS and PROSIS software (Hitachi).

Results

Eight primers were synthesized and used for PCR amplification of the complete coding region of exons six to eight and of two repeated sequences (R4 and R5, Fig. 1).

Detection of gene lesions

The presence of a gene deletion in the four F7 deficient subjects was tested by Southern blotting, using as a probe the cloned amplification products. In *Bgl*II and *Bam*HI digests, which enable us to investigate the gene region including exons two to eight, bands normal in size and intensity were detected (not shown).

For the rapid detection of mutations the amplification products containing the exon regions were studied using perpendicular or parallel TGGE. In three of the four subjects analysis of fragment ef showed altered melting behaviour, including a normal (n) and variant (v) bands (Fig. 2).

To establish the mutation underlying the abnormal melting profile direct sequencing of the PCR products obtained from all subjects was performed. Two mutations were found. A C-to-A transversion (G-to-T in the coding strand) giving rise to the missense mutation TGC to TTC (Cys-310 to Phe) was present in subjects S.M. and R.D. in the homozygous and heterozygous forms

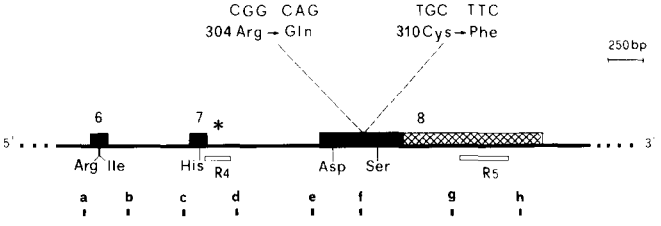

Fig. 1. Schematic diagram of the amplified gene region and localization of mutations and polymorphism. *Numbered black boxes*, exons; *crossed-hatched box*, 3' untranslated region. *a–h*, oligonucleotide primers. This His, Asp and Ser of the catalytic triad and the activation peptide Arg-Ile are shown below and the missense mutations above the exons. *R4, R5*, repeated sequences. The *asterisk* indicates the repeat polymorphism

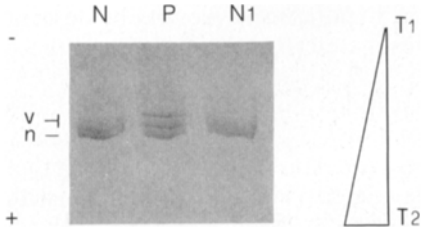

Fig. 2. Temperature gradient gel electrophoresis (TGGE) of amplified fragment ef. The polarity of electrophoresis and the profile of the parallel temperature gradient *(T)* on the plate, are indicated. *n* and *v*, Normal and variant bands. *N, N1*, Normal controls; *P*, patient R.D.

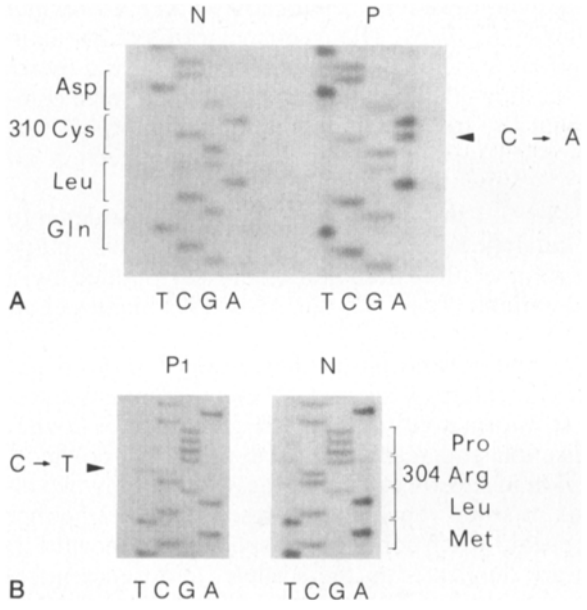

Fig. 3A, B. Direct sequencing of the amplified products. The sequence of the complementary strand is reported; the normally encoded amino acids and the mutated bases are indicated. *N*, Normal; *P*, patient R.D.; *P1*, patient L.M.

respectively (Fig. 3A). A C-to-T transition (G-to-A in the coding strand), causing the substitution of Gln (CAG) for Arg-304 (CGG), was found in the heterozygous form in the subject L.M. (Fig. 3B).

The inheritance of these mutations was studied in the family members by sequencing, TGGE or, since the transition G to A suppresses an *Msp*I site (CCGG), by restriction analysis. The 310 substitution was found, in the heterozygous condition as expected, in the father of patient S.M, the only member of this family available for DNA study. This mutation was not present in two sisters of the compound heterozygous patient R.D. Since F7 activity was reduced to half (52% and 43% respectively) in these subjects, they carry the second mutation not detected in the present study.

In the patient L.M. von Willebrand disease was present in addition to heterozygous F7 deficiency. DNA studies in the family members are compared in Table 1 with the F7 and von Willebrand factor antigen and ristocetin cofactor activity. All sibs had inherited mutation 304 from the father and von Willebrand disease is clearly inherited from the mother. The carriers of the 304 mutation meet the criteria for the heterozygous form of the F7 Padua variant (Girolami et al. 1982), and in particular show a normal F7 antigen level and a normal or low F7 activity using the ox or rabbit brain thromboplastins respectively.

Characterization of a repeat polymorphism

The PCR amplification of two F7 gene regions containing repeated sequences (Fig. 1, R4 and R5) was performed in the F7-deficient subjects and in 20 additional subjects. The R5 analysis failed to show any difference in the subjects examined. In the R4 repeat we have recently found (Marchetti et al. 1991) a polymorphism due to a variable number of repeats. This marker was precisely mapped by restriction analysis. The *Rsa*I sites (Fig. 4), located in the first and last repeat monomers, indicate that the a and b alleles differ in one internal monomer (37 bp in size).

Three F7 deficient subjects were homozygous for the most frequent b allele and all carriers of the 304 mutation were homozygous for the a allele (Table 1). Since F7 and factor X are close on chromosome 13, the inheritance of the polymorphism was in addition studied in a factor X-deficient patient and in his family (Fig. 5). The a allele segregates in this informative family with the altered factor X gene.

Table 1. Molecular and haemostatic studies in family L. F, Father; M, mother; P1, propositus L.M.; S, sisters; N, normal control. n, v, Normal and variant bands in TGGE analysis of fragment ef. R4 P, repeat polymorphism; a and b, alleles (Fig. 5). The 304 codon was established by sequencing. The presence of the *Msp*I site was experimentally determined. FVII:C, FVII activity; FVII:Ag, FVII antigen; vWF:Ag, von Willebrand factor antigen; Ri:Cof, ristocetin cofactor activity of von Willebrand factor

Family L	DNA studies				Haemostatic studies				
	TGGE	304 Codon	*Msp*I	R4 P	FVII:Ag (%)	FVII:C (%)		vWF:Ag (%)	Ri:Cof (%)
						Rabbit brain	Ox brain		
F	n v	Arg Gln	+ −	a a	108	75	100	120	92
M	n	Arg	+ +	a b	84	95	100	46	30
P1	n v	Arg Gln	+ −	a a	70	40	100	46	31
S	n v	Arg Gln	+ −	a a	74	58	100	77	81
S	n v	Arg Gln	+ −	a a	117	51	100	85	66
N	n	Arg	+ +		70–130	80–120	80–120	60–160	60–130

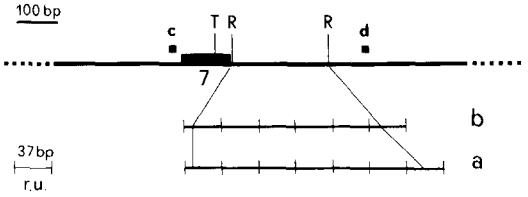

Fig. 4. Map of the repeat polymorphism. *c, d,* Oligonucleotide primers (Fig. 1); *T, TaqI* site; *R, RsaI* site; *a, b,* alleles; *r.u.* repeat unit

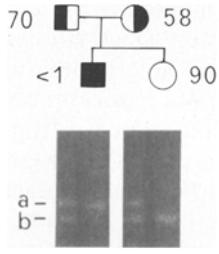

Fig. 5. Inheritance of the repeat polymorphism in a family with a member affected by factor X deficiency. *a, b,* Alleles; ■, affected subject; ◧, ◑, heterozygous parents. The factor X activity values are indicated

Discussion

We studied exons six to eight of the F7 gene, which contain the activation and catalytic domains of the protein and approximately two-thirds (275 codons) of the complete coding sequence. TGGE of crude non-radioactive amplification products, a simplified denaturing technique (Myers et al. 1985) that produces temperature gradients on a plate, was successful in detecting two mutations in exon eight, which represent the molecular defects in four of the seven affected genes.

The Cys-310 to Phe mutation abolishes a disulphide bridge probably with Cys-329, as inferred from the prothrombin structure. This cysteine is conserved in all serine proteases (Leytus et al. 1986) and its substitution is expected to destabilize the tertiary structure, perhaps inducing erroneous folding and formation of illegitimate disulphide bridges.

The similar structure of F7 and factor IX enables us to compare similar mutations found in these genes. The substitution of a positively charged Arg for Cys-336 has been described in haemophilia B with undetectable factor IX activity (Green et al. 1989). In patient S.M., in whom residual F7 activity (FVII:C 4%) and a mild bleeding phenotype are present, the missense mutation Cys-310 to Phe could be less harmful because it preserves a hydrophobic residue.

Transversions are infrequent mutations (5/60 in haemophilia B patients; Koeberl et al. 1990) and thus the occurrence of the G to T transversion (Cys-310 to Phe) in three apparently not related subjects (both parents of S.M. and one parent of R.D.) does not seem to be compatible with the presence of a recurrent mutation. The parents of subject S.M. however are very likely consanguineous, as indicated by their carrier status for Tay-Sachs disease. Informative haplotypes not being available in the F7 gene the hypothesis of identity by descent cannot be further investigated (Reiss et al. 1991) in these patients.

By contrast the transition C to T in CpG dinucleotides and the missense mutation Arg to Gln have frequently been found to be recurrent. Thus it is not surprising that the 304 mutation, associated (James et al. 1991a) with FVII Padua (Girolami et al. 1978), has recently been reported in a patient from a different country (O'Brien et al. 1991).

The Italian propositus was referred for study because of the presence of von Willebrand disease, a rather frequent disorder (Rodeghiero et al. 1987). Its occurrence together with other coagulation gene defects is not unexpected and has been reported together with haemophilia A (Miller et al. 1986). The frequency of FVII Padua in the Piave river valley (Girolami et al. 1982), and therefore of the Arg-304 to Gln mutation, could also be compared with the frequency of an FVII polymorphic variant (Arg-353 to Gln) described recently (Green et al. 1991).

The Arg-304 to Gln mutation has been postulated to reduce catalytic function by removing a positive charge in a portion of the catalytic domain surrounded by a charged variable loop (O'Brien et al. 1991; James et al. 1991b).

The repeat polymorphism characterized in this paper is easily detectable by agarose gel electrophoresis and is the most informative (42%) FVII gene marker found. Amplification and restriction analysis of the polymorphic region in several subjects indicates that only two alleles (six or seven repeats) are present and that neither the 5′ repeat, which contains the splicing site, nor the 3′ repeat, are duplicated in the a allele. Difference in repeat copy number (six or eight monomer repeats) has been reported in two independently derived FVII gene clones (O'Hara and Grant 1988). The eight monomer repeat was not found in this study and could be a rare allele or a cloning artifact, not infrequent in repeated sequences.

The homozygosity for the b allele of three FVII-deficient subjects is associated both with a true homozygous gene defect (S.M.) and with a compound heterozygous condition (R.D.). This polymorphism could be used for linkage studies of diseases and genes located in the terminal region of the long arm of chromosome 13 such as hyperornithinaemia-hyperammonaemia-homocitrullinuria (Valle and Simell 1983) and collagen type IV (COL4A1, COL4A2, Boyd et al. 1986; Killen et al. 1987). Its usefulness was demonstrated in the investigation of a family carrying factor X deficiency, which was not informative for the factor X gene polymorphisms tested.

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