Factor $V_{New\ Brunswick}$: Ala_{221}-to-Val Substitution Results in Reduced Cofactor Activity

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We have characterized the factor V protein and cDNA of a patient displaying factor V deficiency (parahemophilia) and correlated the reduced activity with a missense mutation of Ala_{221}-to-Val. Plasma from the subject individual (C1) presented reduced factor V antigen (39% of normal) that displayed reduced activity (approximately 26% of normal). Factor V purified from this individual by standard techniques shows normal migration on sodium dodecyl sulfate gels and a normal pattern of activation by thrombin. Purified antigen from sibling C2 gives a much reduced specific activity of 263 U/mg (17% of normal). Sibling C3, the mother, and the father have antigen within the normal range (57% to 200%) that has approximately normal specific activity. The cDNA encoding the factor Va heavy and light chains of the subject individual was polymerase chain reaction-amplified and sequenced and revealed an A-to-G substitution at position 3 of codon 51 (silent mutation), a C-to-T substitution in position 2 of codon 221 (Ala_{221}-Val), a T-to-C substitution at position 3 of codon 708 (silent mutation), and a G-to-A substitution at position 1 of codon 2185 (Thr_{2185}-Ala). The latter mutation was also observed in control individuals and is proposed to be a possible polymorphism. Restriction analyses demonstrated the presence of one mutant and one normal allele in the father. The subject individual (C1) and sibling C2 carry only the mutant allele. The mother and sibling C3 carry only the normal allele. The inheritance pattern suggests the presence of a missing or nonexpressed allele in the mother that is passed on to all the siblings. Expression of only the mutant allele by the subject individual (C1) and sibling C2 is consistent with reduced factor V antigen and activity in these patients. We have designated this mutant as Factor $V_{New\ Brunswick}$.

FACTOR V is a 330,000-D molecular weight (M_r) plasma glycoprotein essential to normal blood coagulation.[1] The molecule is composed of multiple domains, with an overall organization of A1, A2, B, A3, C1, C2.[2-4] Removal of the activation peptide, the B domain, by thrombin cleavage yields the fully active cofactor factor Va, which consists of a heavy chain (M_r, 105,000 D) composed of two A domains (A1, A2) noncovalently associated with a light chain (M_r, 74,000 D) consisting of one A domain and two C domains (A3, C1, C2).[5-7] The active factor Va molecule, factor Xa, Ca^{2+}, and phospholipid form the terminal prothrombinase complex of the coagulation cascade.[8-10] Factor Va shares a common domain structure with factor VIIIa, displaying approximately 40% identity in the heavy and light chains.[2-4,11-13]

Human factor V is synthesized as a 2,224-amino acid residue protein that includes a 28-amino acid leader peptide. The factor V message has 6,672 bp of coding sequence, 90 bp of 5′ untranslated sequence, and a 163-bp 3′ untranslated region including a polyA region.[2-4] The factor V gene has been mapped to chromosome 1q21-q25, spans greater than 80 kb, and has 25 exons.[14,15] The size and number of exons in the factor V and factor VIII genes are similar, with the exception of the exon 5 region in factor V, which is encoded by two exons in factor VIII. In both molecules, the entire B domain is encoded by one large exon.[11]

Factor V deficiency (parahemophilia) was first described by Owren in 1947.[16,17] Factor V deficiency normally follows an autosomal recessive inheritance pattern,[18-23] although some possible codominant cases have been reported.[24,25] The occurrence of parahemophilia is low at $1/10^6$, unlike factor VIII deficiency (hemophilia A), which is X-linked and is estimated to occur in 1/10,000 in the general population.[26,27] Factor VIII deficiencies have been studied extensively, while the more rare factor V deficiencies have not undergone extensive investigation. Factor V deficiencies have previously been studied by coagulation and immunologic assays to determine whether genetic defects in patients are due to abnormal molecules lacking procoagulant activity or to decreased quantity of the molecule.[28,29] Here we present a factor V-deficient family that carries a mutant allele containing an Ala_{221}-to-Val substitution. This mutation appears to produce a factor V molecule with reduced cofactor activity and concomitant mild parahemophilia. The inheritance pattern suggests a possible mutation in the second allele of the proband and a sibling, leading to sole expression of the Val_{221} form in these individuals.

MATERIALS AND METHODS

Case history. The proband (C1) first presented at the age of 17 years with a 3-year history of mucus membrane bleeding. Manifestations included epistaxis, hemoptysis, and gum and rectal bleeding. The initial clinical evaluation showed a mildly prolonged prothrombin time (PT) of 13 seconds (normal, 11.2 to 12.5 seconds) and a significantly prolonged activated partial thromboplastin time (APTT) of 49.1 seconds (normal, 26.1 to 37.1 seconds). Coagulation factor assays showed a reduced factor Va activity at 26% (normal, 49% to 150%). All other assays were within the normal levels. Studies of the immediate family members showed normal factor V activity for the proband's 40-year-old father (77%) with no bleeding history,

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whereas that of his 38-year-old mother and his 7-year-old sibling C3 are at the low end of normal, 52% and 54%, respectively. The mother has been diagnosed with menorrhagia, and C3 is reported to have easy bruising. Sibling C2, age 15 years, shows below-normal factor V activity at 27% and has been diagnosed with menorrhagia. All other assays were within normal limits for these family members. Blood samples drawn for factor V purification and DNA and RNA isolation from the patients and family members were obtained after receiving appropriate informed consent.

Materials. All restriction enzymes and DNA modifying enzymes were purchased from Promega Corp (Madison, WI), GIBCO-BRL (Grand Island, NY), or Stratagene (La Jolla, CA). Molecular weight markers were obtained from GIBCO-BRL. Taq DNA polymerase was purchased from Perkin Elmer-Cetus (Norwalk, CT). Human thrombin was purified as previously described.[30] Oligonucleotides were made on an ABI model 381 DNA Synthesizer (Foster City, CA) or purchased from DNA Express (Fort Collins, CO). Nusieve and Seaplaque low melting point agarose were purchased from FMC (Rockland, ME). Acrylamide was purchased from Serva (Paramus, NJ). Bis-acrylamide, nitrocellulose, temed, and ammonium persulfate were purchased from Bio Rad (Richmond, CA). Goat antihorse-peroxidase was purchased from Southern Biotech (Birmingham, AL). All other chemicals were purchased from Sigma (St Louis, MO).

Immunoblotting. Plasma samples from the proband and his family members were blotted using a horse polyclonal factor V antibody. Fifteen microliters of plasma was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4% to 12% gradient gel.[31] The gel was then transferred and blotted as previously described.[32] Fifteen microliters of plasma from each family member was also activated with 0.25 U/mL thrombin for 15 minutes and run along with the untreated samples. To further evaluate the condition of the proband's factor V antigen, factor V was isolated from 5 mL of citrated platelet-poor plasma obtained from the proband and a normal control individual.[28,33] Soybean trypsin inhibitor was added to a concentration of 20 μg/mL, and the plasma sample was stirred on ice. Precipitation of the vitamin K-dependent zymogens in the plasma was performed by the addition of 80 μL of 1 mol/L $BaCl_2$ per milliliter of plasma and 50% polyethylene glycol to a 4% final concentration and centrifugation at 8,000*g*. The supernatant was subsequently applied directly to a 3-mL column of antihuman factor V-1 Sepharose at room temperature. The resin was washed with 20 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L Ca^{++} pH 7.4, and factor V was eluted into 0.5-mL fractions with the same buffer containing 1.7 mol/L NaCl. The factor V eluate (100 μL) was diluted 10-fold and activated with 0.25 U/mL thrombin at 37°C. One hundred-microliter aliquots were removed at various time points from 0 to 10 minutes, quenched into gel sample buffer (62 mmol/L Tris, 2% SDS, 1% 2-mercaptoethanol, and 10% glycerol), and heated at 90°C for 5 minutes. Factor V and factor Va activation fragments were detected by SDS-PAGE followed by Western blotting with horse antihuman factor V polyclonal antibody and the peroxidase ABC detection kit (Vector Labs, Burlingame, CA) using 0.06% 4-chloronapthol, 0.06% H_2O_2 in 20% methanol as a color development substrate. Factor V was also purified from 201 mL of citrated plasma from sibling C2, as previously described.[28,33] The factor V in the peak eluate fraction of the antihuman factor V-1 sepharose was quantitated for mass by absorption at 280 nm using an extinction coefficient of $E^{1\%}_{280} = 9.6$.[28,33] Specific activity of factor V was determined by a two-stage clotting assay.[28] Specific activity of patient factor V was compared with factor V purified from an equivalent volume of normal plasma by the same protocol.

Activity and antigen assays. Factor V antigen quantitation was performed with a competitive radioimmunoassay (RIA), which has been previously described.[29] Factor V activity was measured in a one-stage clotting assay using a normal plasma pool as a standard.[28] The normal plasma pool was assayed with the RIA, and clotting times were converted to μg/mL of factor V. All data are presented as percent of normal using 7 μg/mL as 100% for both the activity and antigen measurements.[29] A two-stage clotting assay was used to assess the activity of factor V after thrombin activation.[28]

Table 1. Oligonucleotide Primers

Primer	Base No.	Sequence
HC-1	16	5'GAGTGTGGTTAGCAGCTC-3'
HC-13	248$_r$	5'CTTGAGTTTGTGGGCTCAGGTC-3'
HC-6	540$_r$	5'ATAGGTGTATTCTCGGCC-3'
HC-5	466	5'GCTTCTTACCTTGACCAC-3'
HC-8	963$_r$	5'-AGCACTGACAAGGGTGAT-3'
HC-7	837	5'-CATGACCACATCAGCTGG-3'
HC-2	1296$_r$	5'-GGTGAAGGACTCATCTTC-3'
HC-3	1149	5'-CATTGCTGCAGAGGAAGT-3'
HC-10	1641$_r$	5'-AGAGGCGATGTCTCTCAT-3'
HC-9	1578	5'-GATGCCCAGTGCTTAACA-3'
HC-11	1989	5'-TGGAAAGAGGCATGAGGA-3'
HC-4	2363$_r$	5'-AGAGCTAGGGCAGTAAGA-3'
LC-1	4782	5'-GACAACATTGCAGCATGG-3'
LBP-2	5469$_r$	5'-TCTCCAAGAACTTCGGGA-3'
LC-6	5377	5'-CCTGTGGACATGAGAGAA-3'
LC-2	5869$_r$	5'-CTGAAGCCTTGATCTGTG-3'
LC-3	5795	5'-CATGGACAGAGACTGTAG-3'
LC-8	6297$_r$	5'-GGGTGTGGAACATCCATT-3'
LC-10	6181	5'-CAGTTTGACCCACCTATTGTGGCTA-3'
LC-9	6596$_r$	5'-CAGCCTGTATGGTTTCCATTCCACT-3'
LC-7	6233	5'-CCAACTCGAGCCTATAAC-3'
LC-4	6796$_r$	5'-GTCTCTTCCAGGGGTTTT-3'

Primers used for amplification of factor V cDNA. Heavy chain and light chain primers correspond to factor V coding region initiating at the base indicated in the second column. Subscript r denotes sequences used for priming in the reverse direction.

Abbreviations: HC, heavy chain; LC, light chain.

Polymerase chain reaction using total RNA. White cells were isolated from the proband, his family, and 25 control individuals using Lymphocyte Separating Media (Organon Technica, Durham, NC) according to the manufacturer's specifications. Total RNA was isolated as previously described.[34] One-microgram samples of total RNA were used for reverse transcription followed by polymerase chain reaction (PCR) using a Gene Amp RNA PCR kit (Perkin Elmer-Cetus). The reverse transcription was performed using an oligo-dT primer to generate cDNA from the total RNA sample. The reaction was conducted in 20-μL volumes containing 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L each deoxynucleoside triphosphate (dNTP), 2.5 μmol/L oligo-dT primer, 20 U RNase inhibitor, and 50 U reverse transcriptase. The reaction mixture was incubated at 42°C for 90 minutes, heated to 99°C for 5 minutes, and then held at 4°C. The reverse transcription reaction was then used for direct amplification. Several pairs of oligonucleotides were used that span the heavy and light chains encoding domains of factor Va (Table 1). The region encoding the B domain corresponds to the activation peptides and is not responsible for cofactor activity.[8] Therefore, this area was not amplified for study. The PCRs were performed in a final volume of 100 μL using the 20-μL reverse transcription reaction, adjusting concentrations to 2 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, and adding 2.5 U Taq polymerase and the appropriate upstream and downstream primers at 15 μmol/L. The reaction mixture was then subjected to 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. The last

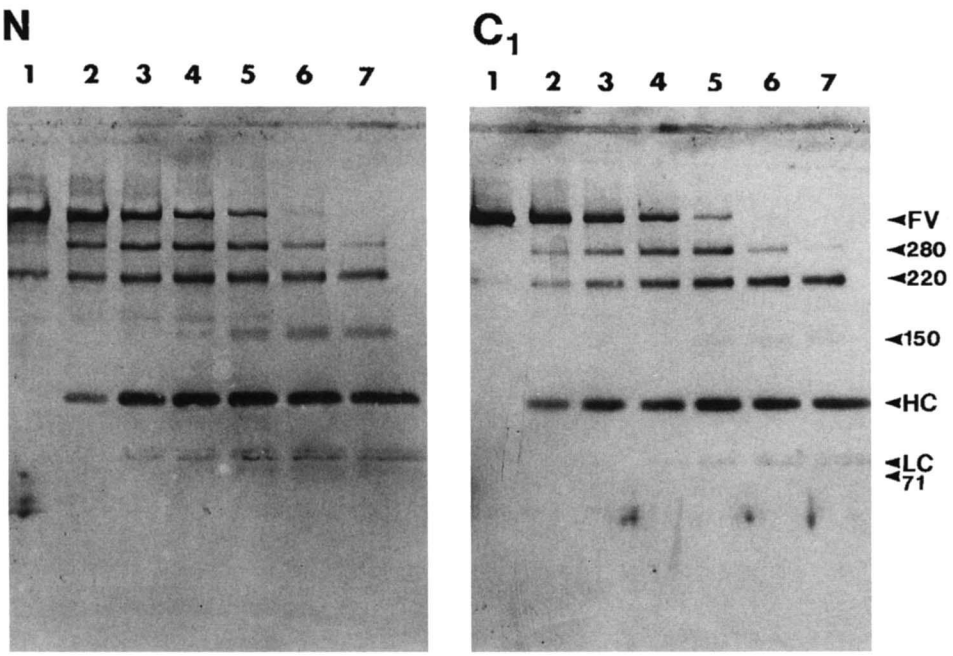

Fig 1. Time-dependent thrombin activation of factor V (FV). A Western blot of purified factor V obtained from a normal control individual (N) and the proband (C1) was performed using an antihuman factor V horse polyclonal antibody. The factor V was activated by thrombin at 0.25 U/mL as described in Materials and Methods. Lane 1, 0 time point; lane 2, 10-second time point; lane 3, 30 seconds; lane 4, 1 minute; lane 5, 2 minutes; lane 6, 5 minutes; and lane 7, 10 minutes. The migration of the fragments is discussed in the text.

cycle ended with an additional elongation step of 10 minutes at 72°C. A second round of amplification was then conducted using semi-nested primers to generate template for sequencing. One microliter of the first-round reaction was used as template in a volume of 100 μL with a concentration of 80 mmol/L Tris-HCl pH 8.3, 20 mmol/L $(NH_4)_2SO_4$, 5 mmol/L $MgCl_2$, 0.5 mmol/L each dNTP, and 1.5 μmol/L of the appropriate oligonucleotides. Temperature cycling was conducted as described above.

Sequencing. The second-round PCRs were run on a 2% low-melting-point agarose gel in a 1:3 ratio of Seaplaque agarose to Nusieve agarose using a 1× TBE buffer (45 mmol/L Tris borate, 1 mmol/L EDTA). The fragments were cut out and isolated with the Geneclean II Plus kit (BIO 101, La Jolla, CA) using the manufacturer's protocol. The fragments were sequenced directly using a Cycle Sequencing kit (ABI). The sequencing reactions were run on a 6.5% polyacrylamide gel with 8.3 mol/L urea, on an ABI 373A DNA sequencer.

RESULTS

Factor V antigen and activity studies. Plasma samples obtained from the family members were immunoblotted with and without thrombin activation to determine if factor V antigen was present and if it was susceptible to thrombin cleavage. All family members had factor V antigen that appeared to activate correctly, generating the appropriate thrombin cleavage fragments. Factor V was subsequently purified from 5 mL of the proband's plasma and subjected to a time-dependent study of activation by thrombin. Figure 1 shows Western blots of purified factor V from the proband (C1) and a normal individual (N). The blots show that factor V antigen is present in the proband's plasma and, when purified, migrates correctly at M_r = 330,000 D (Fig 1, N and C1, lane 1). Thrombin cleavage products of normal and the proband's factor V show equivalent migration on the gel. Thrombin activation proceeds as previously described.[5,7] Initial cleavage of factor V at Arg 709 gives the heavy chain (HC) and the M_r = 280,000 D fragment (Fig 1, lanes 2 through 7). Subsequently, cleavage at Arg 1018 gives the M_r = 220,000 D fragment and the M_r = 71,000 D activation peptide (Fig 1, lanes 3 through 7). A final cleavage at Arg 1545 gives the light chain (LC) and the M_r = 150,000 D activation fragment. The lighter staining intensity of the light chain and the two activation fragments in the patient (Fig 1, C1) is likely due to less antigen on the membrane relative to the normal control (Fig 1, N), and not to a resistance of cleavage by thrombin.

Comparison of antigen and activity determinations in the proband (C1) and sibling C2 showed a reduced activity, while the parent's and sibling C3's antigen and activity measurements were within normal limits (Table 2). The specific activity of purified factor V from sibling C2 was determined by two-stage assay, and the results are summarized in Table 3. Sibling C2 shares identical genotype with C1 and demonstrated a factor V that was clearly activated by thrombin with an activation quotient (AQ) of 48.9, similar to that of normal factor V (AQ = 39.7). The specific activity of the sibling C2 factor V was 17% of normal, which confirms a

Table 2. Factor V Antigen and Activity Values for Factor $V_{New\ Brunswick}$ Family Members

	Normal (%)	M	F	C1	C2	C3
Antigen	57-200	61	101	39	50	61
Activity	50-159	52	77	26	27	54

Antigen was determined by radioimmunoassay as described in Materials and Methods. Activity values were determined by one-stage clotting assays performed with factor V-deficient plasma at the time of sampling from the patients.

Table 3. Specific Activity of Factor V

Factor V	1-Stage (U/mg)	2-Stage (U/mg)	AO
Sibling C2	5.4	263	48.7
Normal	38.0	1,512	39.8

Activity was determined by clotting assay using purified factor V as described in Materials and Methods.

molecular defect that compromises the procoagulant activity of the cofactor.

Amplification and sequencing of the heavy and light chain-encoding regions. To determine if there is a possible mutation in the factor V protein, studies of the proband factor V mRNA were performed. PCR amplification of the cDNA of the factor V light chain-encoding region showed a single base substitution. The A-to-G substitution encodes a Thr-to-Ala missense mutation at amino acid 2185. However, this base substitution was also observed in the contemporaneous control sample and is present in the cDNA and genomic sequences reported by other investigators,[3,14] suggesting that it corresponds to a possible polymorphism. The base reported (A) in our previously published human factor V cDNA sequence study was not observed in the five family individuals and five normal individuals sequenced; therefore, we resequenced the cloned cDNA initially obtained by Jenny et al[2] and found an A, confirming our original published data. We believe that this substitution is most likely a polymorphism or cloning artifact and is not responsible for the decreased factor V activity observed in the proband.

PCR fragments from the factor V heavy chain-encoding area were sequenced and showed three base substitutions. The first is an A-to-G substitution in the codon encoding Gln_{51}, and the second is a T-to-C substitution in the codon encoding Ile_{708}, which has been previously observed by Shen et al.[35] Both of these substitutions were in the wobble position and preserve the coding for the amino acid; neither was observed in the control individual. The third mutation observed was a C-to-T substitution in position 2 of the codon, giving rise to an Ala-to-Val substitution at amino acid 221. This Ala_{221}-to-Val mutation was not seen in five normal individuals sequenced. When the regions of the A1 domain containing the Ala_{221}-to-Val substitution are compared (Fig 2) in bovine and human factor V, one finds complete conservation of identity over the 10-residue sequence surrounding Ala_{221}. In contrast, in this region there is no identity observed between the factor V and factor VIII[2-4,12,36] molecules, except for the conserved Cys residue in all three proteins, which corresponds to amino acid 220 in human factor V.

Restriction analysis. To determine the inheritance pattern of the family, we took advantage of a *Bsp*1286 I restriction site, which is eliminated by the mutation at cDNA position 836 (C-to-T) corresponding to position 2 of codon 221. A 497-bp fragment containing the mutated region was amplified and digested with the restriction enzyme. A normal cleavage pattern generates 190-bp, 181-bp, 89-bp, and 37-bp fragments. The 190-bp and 181-bp fragments migrate as one band when visualized using gel electrophoresis. The fragment containing the mutation generates 218-bp, 190-bp, and 89-bp fragments. The shift of the 181-bp to the 218-bp fragment can be seen on a 5% acrylamide gel. Restriction digests of the PCR fragments of the family members are shown (Fig 3). Lanes 1 and 2 contain ϕX174-*Hae* III molecular weight markers. Figure 3A shows undigested PCR fragments at 497 bp. Figure 3B shows the PCR fragments after digestion with the *Bsp*1286 I restriction endonuclease. In the normal control sample (N), only the 190/181-bp band is seen after digestion (Fig 3B, lane N). Lane C_1 in Fig 3B contains the proband's digested sample, where both the 190-bp and 218-bp bands can be observed. The father (F) and sibling C2 also contain the mutant fragment band (218 bp), while the mother (M) and sibling C3 appear normal. These data show that the mother does not carry the mutant allele, suggesting that the proband inherited the mutant allele from his father. RNA was also isolated from 25 normal control individuals, PCR-amplified, and digested with *Bsp*1286 I. The 218-bp fragment present in the mutant individuals was not observed in any of the 50 control alleles investigated (data not shown).

Sequence of mutation in immediate family. To confirm the inheritance pattern, we conducted sequence analysis on the available family members (Fig 4). The proband (C1) shows only the mutant allele present from the amplified mRNA. The father appears heterozygous, having both the mutant and the normal alleles, while the mother appears normal. Sibling C2 is identical to the proband, showing only the mutant allele, while sibling C3 shows only the normal allele. All family members were amplified and sequenced at least twice. Two different different blood samples were obtained for the proband and the sibling C2; these were both amplified and sequenced at least twice. Sequencing of the silent mutations in these family members shows the same pattern, confirming that these mutations appear to be allelic to the Ala_{221}-to-Val substitution.

The Thr_{2185}-to-Ala polymorphism observed in the light chain of the factor V cDNA molecule is present in every family member, along with the normal control individual.

DISCUSSION

We have identified two missense point mutations in factor V that correspond to a Thr_{2185}-to-Ala possible polymorphism

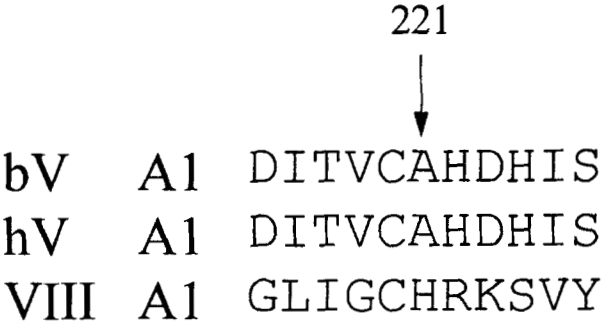

Fig 2. Comparison of the amino acid sequence of the A1 domain of human (hV) and bovine (bV) factor V and human factor VIII (VIII) corresponding to the region that contains the Ala_{221}-to-Val substitution.

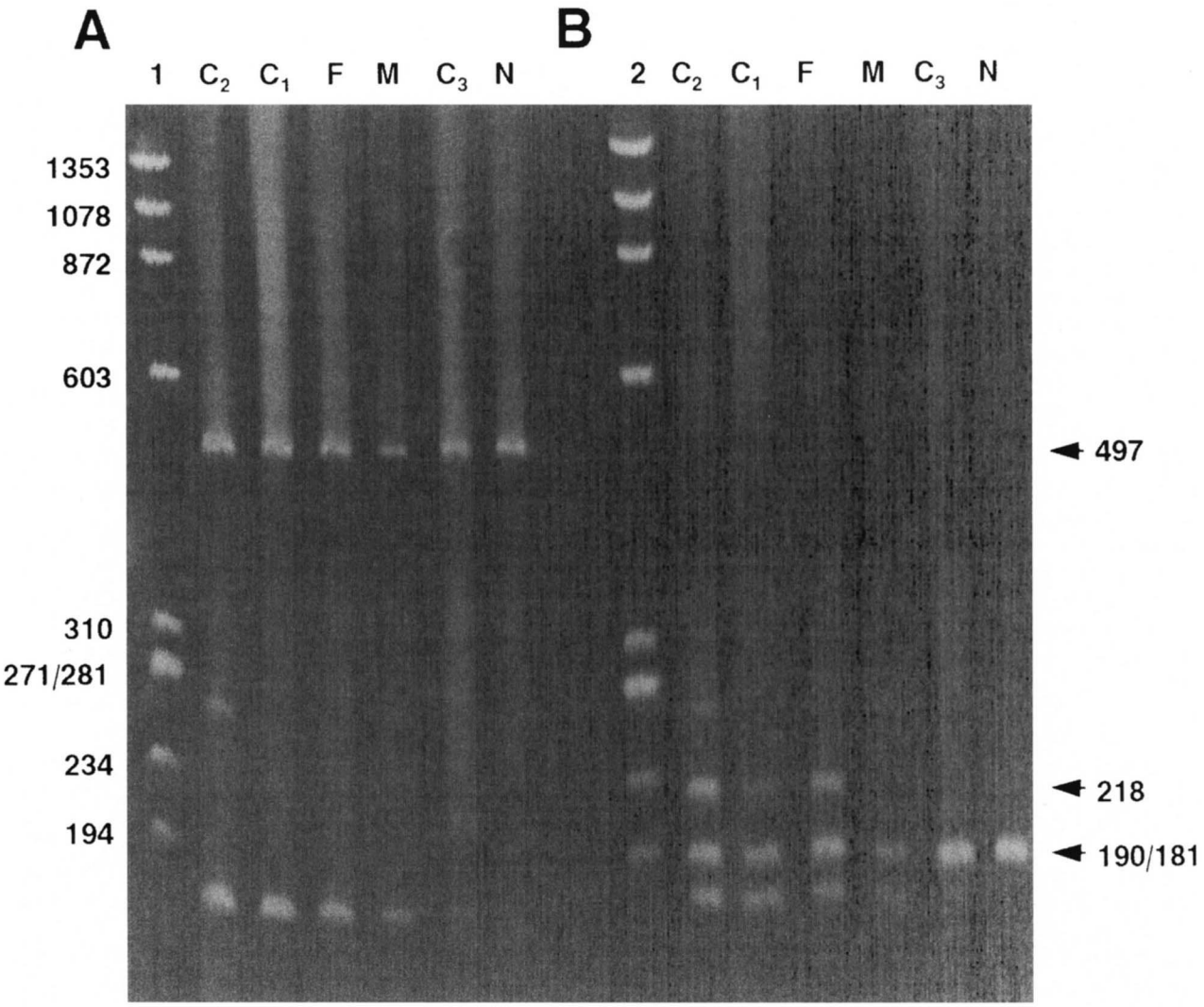

Fig 3. Restriction analysis of PCR products from proband's immediate family members. The restriction enzyme *Bsp* 1286 I was used to digest the 497-bp PCR fragments shown in panel A. Panel B shows the results of the restriction digest. The shift of the 181-bp band to a 218-bp band can be seen in the lanes marked C_1, C_2, and F. The normal restriction pattern of a 181/190-bp band can be seen in the lanes marked N, M, and C_3. Lanes are abbreviated as follows: F, father; M, mother; C_1, sibling C1; C_2, sibling C2; and C_3, sibling C3; N, normal. Lanes 1 and 2 contain molecular weight markers.

in the C2 domain and an Ala_{221}-to-Val substitution in the A1 domain of factor V. The antigen/activity data along with restriction analyses and cDNA sequencing support an inheritance pattern as shown in Fig 5. These results suggest that there is a missing or unexpressed allele that is inherited by the proband (C1) and C2 from their mother. C1 has the missense mutant allele, which he inherited from his father, and a missing or unexpressed allele, which he inherited from his mother. The father is heterozygous, having the missense mutant allele and a normal allele, while the mother has a normal allele and a possible missing or unexpressed allele. C2 has a similar inheritance pattern as the proband, and C3 is either homozygous normal or is similar to the mother. The similarity of activity (61%) and antigen (52% *v* 54%) levels between the mother and sibling C3 suggests that C3 is similar to the mother, having inherited the mutant allele from her mother and a normal copy from her father.

The mutant allele appears to code for a factor V antigen with reduced activity. Analysis of purified factor V (factor $V_{New\ Brunswick}$) from patients C1 and C2 is consistent with the conclusion that the Ala_{221}-to-Val substitution leads to compromised cofactor activity. Factor $V_{New\ Brunswick}$, when activated by thrombin, generates fragments with normal mobility on SDS-PAGE, and a near 50-fold increase in cofactor activity is observed. The specific activity of purified factor $V_{New\ Brunswick}$ is 17% of normal. Thus, the lower activity observed for this molecule is most likely due to a non-ideal interaction of factor $V_{New\ Brunswick}$ with factor Xa, and not a result of incomplete thrombin activation.

The missense Ala_{221}-to-Val mutation is adjacent to a Cys residue that is conserved in bovine and human factor V and in human factor VIII.[37] The disulfide bonds of bovine factor V have been determined, and the bovine Cys that corresponds to Cys 220 is involved in a disulfide bridge with Cys

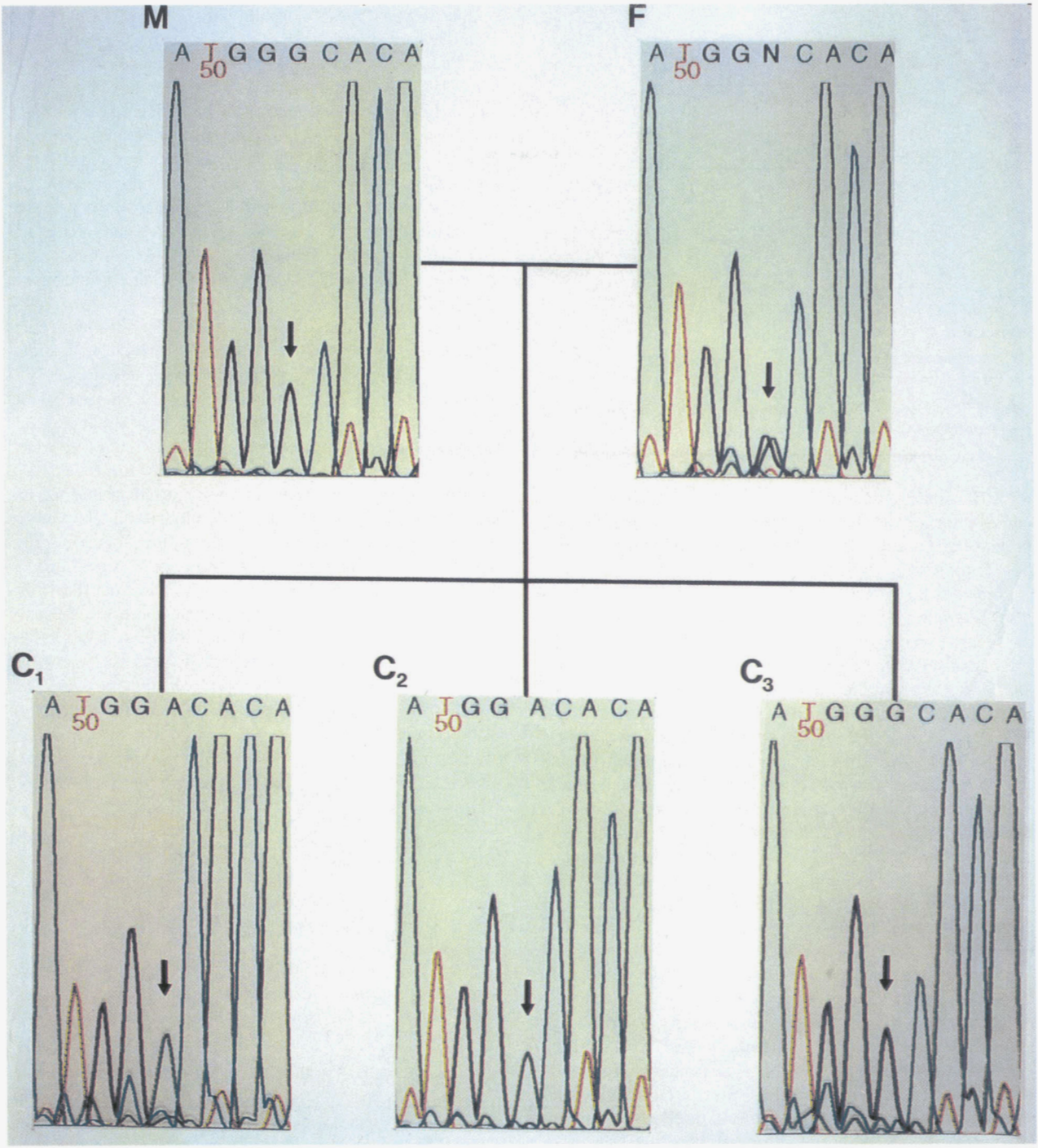

Fig 4. Sequence of the PCR fragment surrounding the Ala_{221} codon in the family members. The C-to-T substitution causing the Ala_{221}-to-Val amino acid change is observed in C1 and C2. The mother (M) and sibling C3 display the normal base in the codon, whereas the father (F) shows both the normal and the substituted base. The arrow indicates the base substitution.

301, forming a large β loop in the A1 domain. All Cys residues involved in disulfide bonding in bovine factor V are conserved in human factor V and factor VIII, suggesting that the disulfide bridges in all three proteins are identical. Previously published data have shown that a monoclonal antibody that binds the A1 domain of factor V inhibits factor Va/factor Xa interactions.[38] Although the exact epitope of this interaction has not been determined, previous data coupled to that of the present study suggest that the Ala_{221}-to-Val substitution may interfere in factor Va binding of factor Xa. If this area in the factor V cofactor is involved in serine protease binding, then differences between factor V and fac-

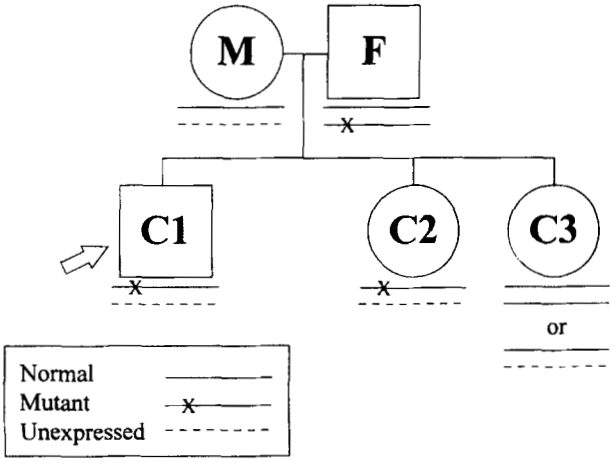

Fig 5. Proposed inheritance pattern for New Brunswick family: (——), normal; (X), mutant; (- - -), unexpressed alleles.

tor VIII would be expected, leading to specificity of each cofactor for its appropriate serine protease; indeed, the two proteins are entirely dissimilar in this region, with the exception of the Cys_{220}.

Although the mutation in the unexpressed allele has not been determined, it seems clear from the data presented that the Ala_{221}-to-Val substitution is responsible for the reduced activity observed in the proband C1 and his sibling C2. An Ala_{221}-to-Val mutation that causes mild/moderate hemophilia A has been observed in the A2 domain of factor VIII.[39,40] Although the exact functional significance for this mutation has not been determined, it is interesting to note that this relatively conservative substitution has a similar effect on factor VIIIa cofactor activity. An individual with the Ala_{221}-to-Val factor VIII mutation has an antigen level of 25% and an activity of 14%, similar to the proband in this study with a 39% antigen level and 26% activity.

The bleeding diathesis of parahemophilia consists of echymosis, epistaxis, and menorrhagia. Bleeding can also occur after trauma or surgery. Heterozygotes for factor V gene defects are usually asymptomatic and have moderately low or low normal levels of factor V activity. Some studies, however, have shown varying expressivity in heterozygotes.[21-23] The normal ranges for factor V antigen and activity are 50% to 200% and 50% to 159%, respectively. This wide normal range may help to account for the differential expression seen in heterozygotes. Two of the three suspected heterozygotes in this study vary from no bleeding (father) to menorrhagia or easy bruising (mother and C3, respectively). In the two compound heterozygotes (C1 and C2), it is interesting that the proband C1 has much more severe bleeding tendencies than that of his sister C2. The expressivity difference in individuals has made determining inheritance patterns difficult in the past. The ability to observe molecular defects directly will undoubtedly help in the understanding of this deficiency and the effects different mutations have on different individuals.

Of particular interest in this study is the father of the proband. Although this individual has the mutant genotype, his phenotype appears normal, with 101% of normal factor V antigen levels and 77% of normal factor V activity. This individual has experienced no abnormal bleeding problems. The Ala_{221}-to-Val mutation would probably have gone undetected if not for the presence of the null allele in the proband, which appears to have been inherited maternally. The presence of this mutation, while unfortunate for the affected individuals, was fortuitous for the study of the biochemistry of blood coagulation in that this study shows the presence of underlying mutations in the proteins involved in coagulation that can remain undetected yet have serious consequences in affected individuals under certain circumstances of inheritance.

To our knowledge, this is the first-described molecular defect in the factor V molecule that leads to reduced coagulation cofactor activity of the factor Va molecule. Recently, a mutation has been described that occurs at an activated protein C cleavage site Arg_{506}-to-Gln.[41,42] This mutation leads to an increase in thrombosis due to the inability of activated protein C to rapidly inactivate the factor V molecule.[43] We have also discovered a possible polymorphism that we observed in several normal individuals along with all members of this family. Previously, a *Pst* I restriction fragment polymorphism has been described in the factor V gene,[44] and, as mentioned earlier, the Ile_{708} silent mutation has been observed elsewhere.[35] To our knowledge, these are the only polymorphisms described for factor V. Further research into this molecule's natural defects will help to define regions of the molecule that are important to its function and will undoubtedly help in the understanding of the overall function of this cofactor.

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REFERENCES

1. Mann KG, Nesheim ME, Tracy PB: Molecular weight of undegraded plasma factor V. Biochemistry 20:28, 1981

2. Jenny R, Pittman D, Toole J, Kriz R, Aldape R, Hewick R, Kaufmann R, Mann KG: Complete cDNA and derived amino acid sequence of human FV. Proc Natl Acad Sci USA 84:4846, 1987

3. Kane WH, Davie EW: Cloning of a cDNA coding for human factor V, a blood coagulation factor homologous to factor VIII and ceruloplasmin. Proc Natl Acad Sci USA 83:6800, 1986

4. Kane WH, Ichinose A, Hagen FS, Davie EW: Cloning of cDNAs coding for the heavy chain region and connecting region of human factor V, a blood coagulation factor with four types of internal repeats. Biochemistry 26:6508, 1987

5. Suzuki K, Dahlback B, Stenflo J: Thrombin-catalyzed activation of human coagulation factor V. J Biol Chem 257:6556, 1982

6. Foster WB, Nesheim ME, Mann KG: The factor Xa-catalyzed activation of factor V. J Biol Chem 258:13970, 1983

7. Monkovic DD, Tracy PB: Activation of human factor V by factor Xa and thrombin. Biochemistry 29:1118, 1990

8. Mann KG, Jenny RJ, Krishnaswamy S: Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. Ann Rev Biochem 57:915, 1988

9. Krishnaswamy S, Church WR, Nesheim ME, Mann KG: Activation of human prothrombin by human prothrombinase: Influence

of factor Va on the reaction mechanism. J Biol Chem 262:3291, 1987

10. Mann K, Nesheim M, Church W, Haley P, Krishnaswamy S: Surface dependent reactions of the vitamin K-dependent enzyme complexes. Blood 76:1, 1990

11. Kane WH, Davie EW: Blood coagulation factors V and VIII: Structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood 71:539, 1988

12. Toole JJ, Knopf JL, Wozney JM, Sultman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Shoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM: Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature 312:342, 1984

13. Vehar G, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood W, Harkins R, Tuddenham EGD, Lawn R, Capon D: Structure of human factor VIII. Nature 312:337, 1984

14. Cripe LD, Moore, Kane WH: Structure of the gene for human coagulation factor V. Biochemistry 31:3777, 1992

15. Dahlback B, Hansson C, Islam MQ, Szpirer J, Szpirer C, Lundwall A, Levan G: Assignment of gene for coagulation factor V to chromosome 1 in man and to chromosome 13 in rat. Somat Cell Mol Genet 14:509, 1988

16. Owren PA: The coagulation of blood. Investigations on a new clotting factor. Acta Med Scand 194:1, 1947

17. Owren PA, Cooper T: Parahemophilia. Arch Intern Med 95:194, 1955

18. Terheggen HG: Faktor V-Mangel bei einem 8 monate alten Madchen. Monatsschr Kinderheilkd 119:627, 1971

19. Seeler RA: Parahemophilia: Factor V deficiency. Semin Thromb Hemost 56:119, 1972

20. Mitterstieler G, Muller W, Geir W: Congenital factor V deficiency: A family study. J Haematol 17:159, 1969

21. Lopez V, Pflugshaupt R, Wirthner H, Butler R: Herekitarer Faktor V-Mangel (Parahamophilie) in einer Schweizer Familie. Schweiz Med Wochenschr 99:1354, 1969

22. DeVries A, Matoth Y, Sharmir Z: Familial congenital labile factor deficiency with syndactilism. Acta Haematol 5:129, 1951

23. Brink AJ, Kingsley CS: A familial disorder of blood coagulation due to deficiency of the labile factor. Q J Med 21:19, 1952

24. Davie EW, Fujikawa K, Kurachi K, Kisiel W: The role of serine proteases in blood coagulation cascade. Adv Enzymol 48:277, 1975

25. Hoffman M, Roberts HR: Hemophilia and related conditions—Inherited deficiencies of prothrombin (factor II), factor V, and factors VII to XII, in Williams WH, Beutler E, Lichtman M, Coller BS, Kipps TJ (eds): Williams Hematology. New York, NY, McGraw-Hill, 1995, p 1413

26. Hoyer LW: Molecular pathology and immunology of factor VIII (hemophilia A and factor VIII inhibitors). Hum Pathol 18:153, 1987

27. Sadler JE, Davie EW: Hemophilia A, hemophilia B, and von Willebrand's disease, in Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW (eds): The Molecular Basis of Blood Diseases. Philadelphia, PA, Saunders, 1987, p 575

28. Nesheim ME, Katzmann JA, Tracy PB, Mann KG: Factor V, in Lorand L (ed): Methods in Enzymology, Proteolytic Enzymes, Part C. New York, NY, Academic, 1981, p 249

29. Tracy PB, Eide LL, Bowie EJW, Mann KG: Radioimmunoassay of factor V in human plasma and platelets. Blood 60:59, 1982

30. Bajaj SP, Rapaport SI, Prodanos C: A simplified procedure for purification of human prothrombin, factor IX and factor X. Prep Biochem 11:397, 1981

31. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

32. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979

33. Katzman JA, Nesheim ME, Hibbard LS, Mann KG: Isolation of functional human coagulation factor V by using a hybridoma antibody. Proc Natl Acad Sci USA 78:162, 1981

34. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

35. Shen NL, Fans T, Pyati J, Graff R, LaPolla RJ, Edgington TS: The serine protease cofactor factor V is synthesized by lymphocytes. J Immunol 150:2992, 1994

36. Guinto ER, Esmon CT, Mann KG, MacGillivray RTA: The complete cDNA sequence of bovine coagulation factor V. J Biol Chem 267:2971, 1992

37. Xue J, Kalafatis M, Silveira JR, Kung C, Mann KG: Determination of the disulfide bridges in factor Va heavy chain. Biochemistry 33:13109, 1994

38. Kalafatis M, Xue J, Lawler CM, Mann KG: Contribution of the heavy and light chains of factor Va to the interaction with factor Xa. Biochemistry 33:6538, 1994

39. Higuchi M, Antonarakis SE, Kasch L, Oldenburg J, Economou-Petersen E, Olek K, Arai M, Inaba H, Kazazian HH: Molecular characterization of mild-to-moderate hemophilia A: Detection of the mutation in 25 of 29 patients by denaturing gradient gel electrophoresis. Proc Natl Acad Sci USA 88:8307, 1991

40. Tuddenham EGD, Schwaab R, Seehafer J, Millar DS, Gitschier J, Higuchi M, Bidichandani S, Connor JM, Hoyer LW, Yoshioka A, Peake IR, Olek K, Kazazian HH, Lavergne J-M, Giannelli F, Antonarakis SE, Cooper DN: Hemophilia A: Database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene, 2nd ed. Nucleic Acids Res 22:3511, 1994

41. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Rhonde H, van der Velden PA, Reitsma PH: Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 369:64, 1994

42. Voorberg J, Roelse J, Koopman R, Buller H, Berends F, ten Cate JW, Mertens K, van Mourik JA: Association of ideopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 343:1535, 1994

43. Kalafatis M, Rand MD, Mann KG: The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem 269:31869, 1994

44. McAlpine PJ, Dixon M, Guinto ER, MacGillivray RTA: A PstI polymorphism in the human coagulation factor V (F5) gene. Nucleic Acids Res 18:7471, 1990