Mutation in blood coagulation factor V associated with resistance to activated protein C

**Rogier M. Bertina*, Bobby P. C. Koeleman*,
Ted Koster†, Frits R. Rosendaal*†,
Richard J. Dirven*, Hans de Ronde*,
Pieter A. van der Velden* & Pieter H. Reitsma***

* Hemostasis and Thrombosis Research Center, and
† Department of Clinical Epidemiology, University Hospital,
Bldg 1-C2R, PO Box 9600, 2300 RC Leiden, The Netherlands

ACTIVATED protein C (APC) is a serine protease with potent anti-coagulant properties, which is formed in blood on the endothelium from an inactive precursor[1]. During normal haemostasis, APC limits clot formation by proteolytic inactivation of factors Va and VIIIa (ref. 2). To do this efficiently the enzyme needs a non-enzymatic cofactor, protein S (ref. 3). Recently it was found that the anticoagulant response to APC (APC resistance)[4] was very weak in the plasma of 21% of unselected consecutive patients with thrombosis[5] and about 50% of selected patients with a personal or family history of thrombosis[6,7]; moreover, 5% of healthy individuals show APC resistance, which is associated with a sevenfold increase in the risk for deep vein thrombosis[5]. Here we demonstrate that the phenotype of APC resistance is associated with hetero-zygosity or homozygosity for a single point mutation in the factor V gene (at nucleotide position 1,691, G → A substitution) which predicts the synthesis of a factor V molecule (FV Q506, or FV Leiden) that is not properly inactivated by APC. The allelic fre-quency of the mutation in the Dutch population is ~2% and is at least tenfold higher than that of all other known genetic risk factors for thrombosis (protein C (ref. 8), protein S (ref. 9), antithrombin[10] deficiency) together.

The responsiveness of plasma to APC is measured as the ratio of two activated partial thromboplastin times, one in the pres-ence of APC and one in its absence[4,5,7]. This APC-sensitivity ratio (APC-SR) is normalized to the ratio obtained with a refer-ence plasma (n-APC-SR). Resistance to APC is defined by a n-APC-SR < 0.84 (1.96 s.d. below the mean n-APC-SR in 100 healthy controls, after outlier removal).

Analysis of the parentships of 14 unrelated APC-resistant patients led to the concept of a familial form of APC resistance (or deficiency of APC cofactor II[4]) in which homozygotes and heterozygotes can be identified on the basis of the n-APC-SR (Fig. 1 legend). Further support for this came from mixing equal volumes of normal plasma and plasma from a patient classified as homozygous cofactor II-deficient (n-APC-SR, 0.38), which gave a n-APC-SR of 0.57 (Fig. 1a). This is identical to the ratio for plasma from patients heterozygous for the deficiency (mean n-APC-SR, 0.58). Mixing the plasma of four unrelated homo-zygous APC cofactor II-deficient patients (mean n-APC-SR, 0.40) did not alter the ratio, indicating that in all four patients the same plasma protein was missing or defective (see also refs 4 and 7).

To investigate whether APC cofactor II activity is a feature of one of the known blood coagulation proteins, APC cofactor II was assayed in a series of plasmas deficient in a single protein (Fig. 1b). All contained normal levels of APC cofactor II (60–155%) apart from plasma deficient in factor V (<5%). Addition of isolated human factor V to factor V-deficient plasma intro-duced factor V coagulant activity and APC cofactor II activity, suggesting that the latter is related to factor V (see also ref. 11).

Independent support for the identity of factor V with APC cofactor II came from linkage studies in a large family with APC resistance (Fig. 2a). The human locus for the factor V gene (F5) has been mapped to chromosome 1 (1q21-25)[12]. There are no reports of polymorphic F5 markers[13-17] that can be amplified by polymerase chain reaction (PCR). Therefore, we tested the
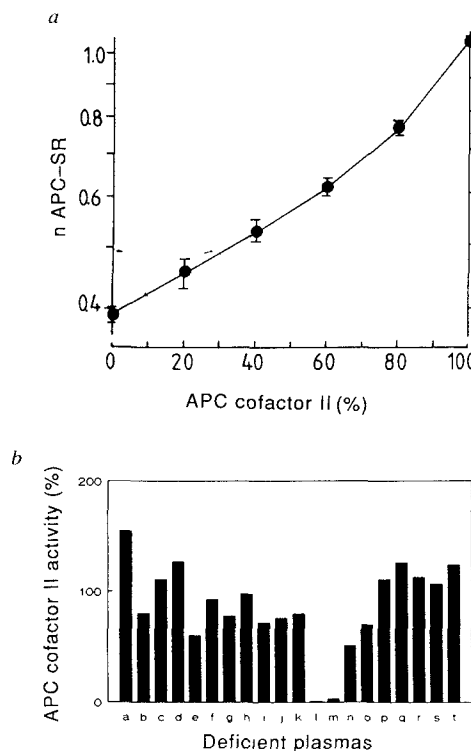

FIG. 1 Measurement of APC-cofactor II levels in plasma. a, Calibration curve for the assay of APC-cofactor II activity in plasma. APC cofactor II refers to the hypothetical new cofactor of APC[4] which is missing or defective in individuals with APC resistance. n-APC-SRs were measured in dilutions of normal plasma (100% APC cofactor II) in plasma of a patient homozygous-deficient in APC cofactor II (0% APC cofactor II). The curve is the result of nine different experiments. The classifica-tion as homozygous- or heterozygous-deficient in APC cofactor II is based on the results of parentship analysis for 14 probands with APC resistance (n-APC-SR<0.84). For 2 probands (n-APC-SR, 0.38/0.41), both parents were APC resistant (mean n-APC-SR 0.55); for 11 probands (mean n-APC-SR 0.57) one of the parents was APC resistant (mean n-APC-SR 0.59) whereas the other was not (mean n-APC-SR 0.96); for one proband (n-APC-SR 0.74), neither parent was affected (n-APC-SR 0.96/0.99). We propose that individuals can be classified as homozyg-otes or heterozygotes for APC cofactor II deficiency on the basis of their n-APC-SR (homozygotes: mean 0.40, $n=2$; heterozygotes: mean 0.58, range 0.51–0.67, $n=26$). b, APC cofactor II activity levels in plasmas deficient (<5%) in a single coagulation factor. Plasmas were either from patients with a congenital deficiency (a, g, f, m, g, r, s, t) or prepared by immunodepletion (b, c, d, e, j, h, i, k, l, p). Plasmas were deficient in factor II (a), factor VII (b), factor IX (c), factor X (d), factor XI (e), factor XII (j), factor XIII (g), protein C (l), protein S (i), β2-glycoprotein (j), antithrombin (k), factor V (l, m), factor VIII (p, q) or von Willebrand factor (r, s, t). Factor V-deficient plasma (m) was supplemented with two different concentrations, 54% (n) and 90% (o), of purified human factor V (Serbio, Gennevilliers, France), dialysed against 20 mM sodium citr-ate, 150 mM NaCl, 4 mM $CaCl_2$ and tested for APC cofactor II activity. METHODS. The APC-SR was calculated from the results of two activated partial thromboplastin times, one measured in the presence of APC and one in its absence, as before[5]. The n-APC-SR was calculated by dividing the APC-SR for the test sample by the APC-SR for pooled normal plasma. APC cofactor II activity was measured by reading the n-APC-SR for two different dilutions (1:1, 3:4) of the test plasma in APC cofactor II-deficient plasma on a calibration curve as shown in a.

segregation of microsatellite markers for several loci in the 1q21–25 region (Fig. 2b) in the family. Significantly positive results were obtained only for locus $D1S61$ (Z_{max} 7.27 at $\theta = 0.00$), which is located within 4 cM of the $F5$ locus (see table in Fig. 2c).

We then searched for an associated mutation(s) in the factor V gene in regions containing the putative APC-binding site (corresponding to amino-acid residues 1,865–1,874)[18,19] and the putative APC cleavage site (Arg 506)[13,20]. Ectopic transcripts of the factor V gene from blood lymphocytes were used for first-strand synthesis of complementary DNA and subsequent amplification of the two regions coding for the APC binding and cleavage sites. Direct sequencing of the PCR fragments revealed that two patients, classified as homozygous for deficiency of APC cofactor II, were both homozygous for a guanine to adenine substitution at nucleotide 1,691 ($1,691G \rightarrow A$) (Fig. 3a). This mutation predicts the replacement of Arg 506 (CGA) by Gln (CAA) (FV Q506 or FV Leiden). No other sequence abnormalities were detected in 225 base pairs (bp) incorporating 1,691 A or in 275 bp around the region coding for the putative APC-binding site (Fig. 3b).

If cleavage after Arg 506 is necessary for inactivation of human factor Va by APC, introduction of a glutamine at posi-

tion 506 should prevent inactivation. During coagulation, factor V is first activated by factor Xa (with formation of a 105/220K heterodimer[21]) and then processed by thrombin (with formation of a 105/74K heterodimer[22,23]). We find that replacement of Arg 506 by Gln prevents inactivation by APC of factor Va formed after addition of factor Xa (Fig. 3c), but not that of factor Va formed after addition of α-thrombin (data not shown).

As two unrelated APC-resistant patients were homozygous for the same mutation, this alteration may predominate in other APC-resistant patients. We therefore designed a test to screen genomic DNA for the presence of the $1,691G \rightarrow A$ substitution. The mutation is located in exon 10, 11 nucleotides 5' of the start of intron 10, and as only the first 8 nucleotides of intron 10 have been sequenced[16], we generated more intron 10 sequence by heminested reverse PCR[24] and then designed primers for the amplification of two overlapping genomic fragments for use in genotyping.

The 267-bp fragment was digested with MnlI to establish whether the allele was normal (G at 1,691) or mutated, and hybridization of the 222-bp fragment with oligonucleotides specific for each allele was used to confirm the presence of adenine at nucleotide 1,691. Using this approach, we investigated all the members of the pedigree shown in Fig. 2a. There was com-
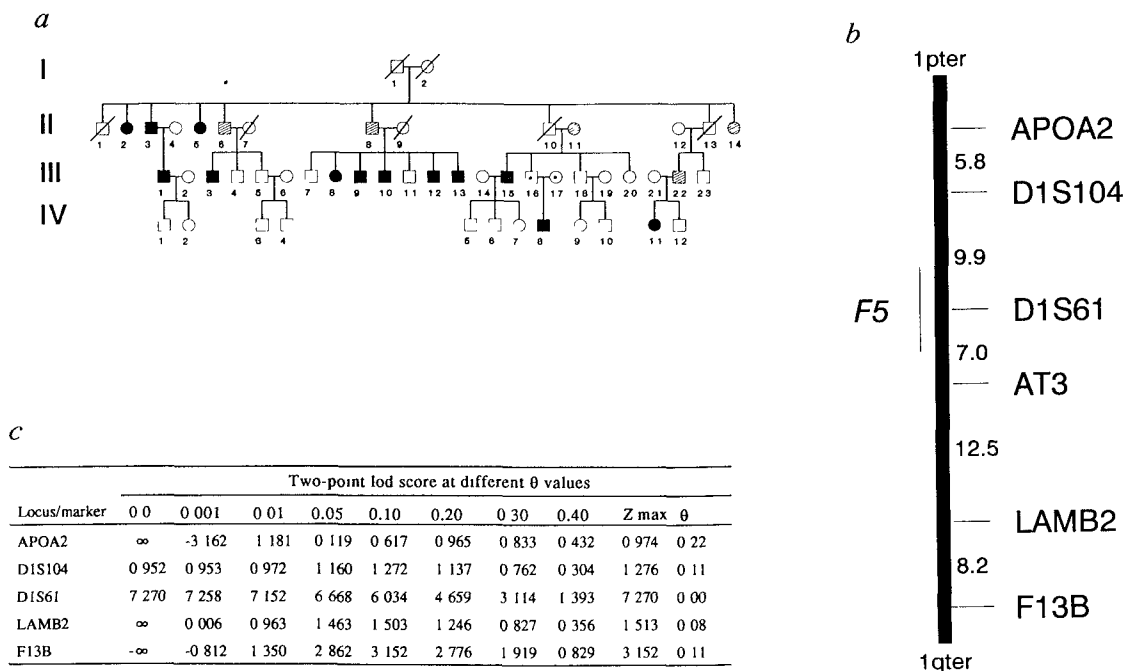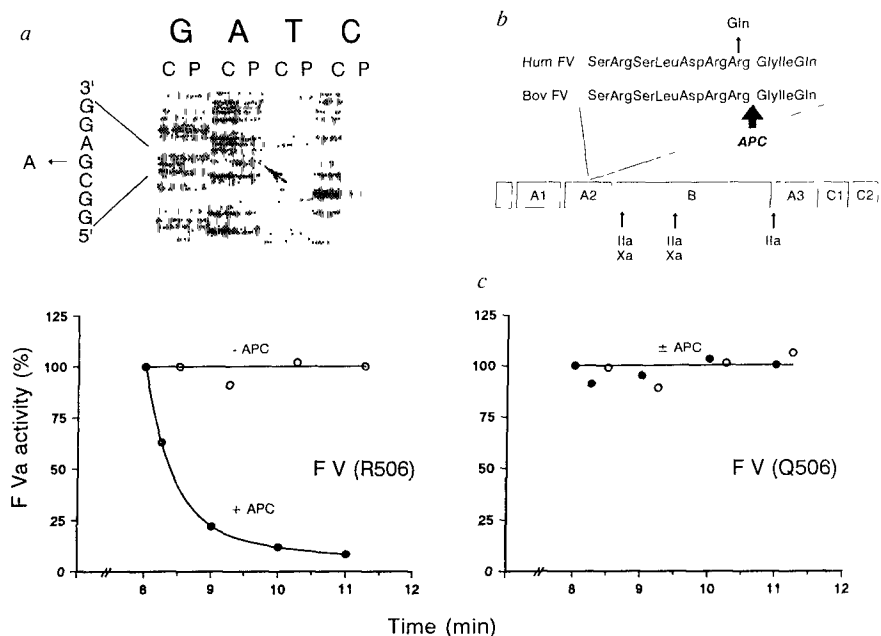
a

b

FIG. 2 Linkage analysis in a family with APC-resistance. a, Pedigree of a family with APC-resistance (or APC cofactor II deficiency). This pedigree forms part of a larger pedigree originally identified in our laboratory because of symptomatic type-I protein C deficiency. ●, ■, individuals with n-APC-SR < 0.84 (mean 0.65; range 0.59–0.71, n = 13); ○, □, individuals with n-APC-SRC > 0.84 (mean 1.03; range 0.87–1.29; n = 20); ⊘, ▨, patients treated with oral anticoagulants (measurement of n-APC-SR in these patients is not meaningful), ○, □, individuals not tested. History of venous thrombosis: II 3, 6, 8 and 14, and III 1, 9, 20 and 22; carriers of the protein C mutation (residue at position 230, R → C): II 3, 6, 8 and 14; III 1, 5, 7, 9, 12, 18, 20, 22 and 23 and IV 1, 3, 4, 10 and 12. b, Integrated genetic linkage map of the q21–25 region of chromosome 1. The relative positions of the loci APOA2, D1S104, D1S61, AT3, LAMB and F13B were derived from the NIH/CEPH Collaborative Mapping Group linkage map[25]. The genetic distance between adjacent loci is given in cM. The F5 locus was placed on this map within 4 cM of the D1S61 locus by studying the segregation of markers for the F5 and D1S61 loci in 3 CEPH families informative for both markers (in 55 meioses, no recombination between these two loci was observed; Z_{max} 16.6 at $\theta = 0.00$). c, Pairwise lodscores of APC-resistance with chromosome 1 markers. All available individuals of the pedigree shown in a were analysed. Oligonucleotide sequences for mar-

kers for the loci ApoA2, D1S104, D1S61, LAMB and F13B are available from the Genome Data Bank. Primers were obtained from the Dutch primer base Three different polymorphic markers for the AT3 locus were not informative in this family Two-point linkage analysis was performed using the MLINK program from the LINKAGE package version 5.3 (from J Ott). Sex-averaged lodscores are shown.
METHODS Microsatellite markers for ApoA2, D1S104, D1S61, LAMB and F13B were amplified by PCR. Conditions: 50 mM NaCl, 10 mM Tris-HCl, pH 9.6, 10 mM MgCl₂, 0.01% BSA, 200 μM dGTP, dATP and dTTP, 20 μM dCTP, 0.7 μCi [α-^{32}P]dCTP, 0.43 U Taq polymerase (Cetus), 50 ng of each primer and 30 ng genomic DNA 27 Cycles were run at 94 °C (1 min), 55 °C (2 min) and 72 °C (1 min), with a final elongation step of 10 min PCR products were separated on a 6% denaturing polyacrylamide sequence gel, after which gels were dried and exposed to X-ray film. F5 polymorphisms: A 636-bp fragment from exon 13 of the factor V gene[16] was amplified by PCR using the primers 5'-TGCTGACTATGATT-ACCAGA-3' (PR-766, nucleotides 2,253-2,272; ref 13) and 5'-GAGT-AACAGATCACTAGGAG-3' (PR-768, nucleotides 2,870–2,899; ref. 13). For PCR conditions, see legend to Fig. 4. Restriction with HinfI detects a C/T dimorphism at nucleotide 2,298 (C: 0.68; T: 0.32) and a rare A/G dimorphism at nucleotide 2,411 (A, 0.98; G, 0 02). None of these markers was informative in the pedigree in a.
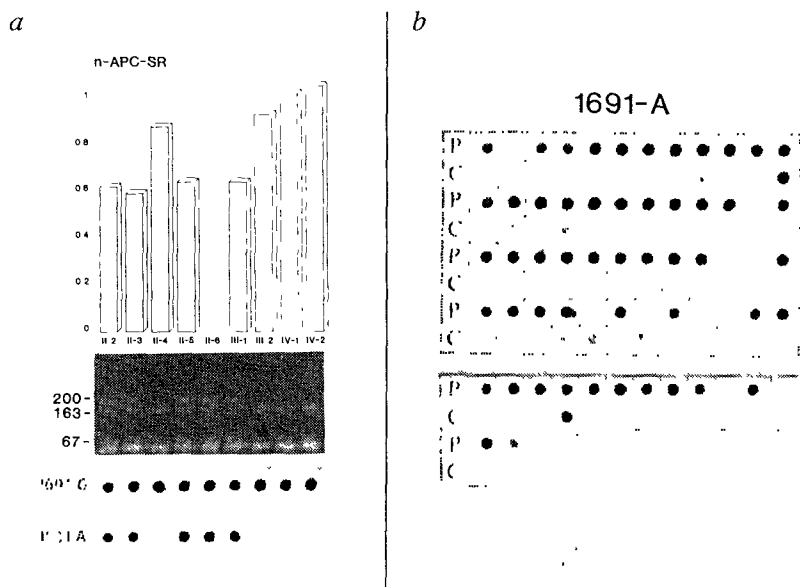
c

Locus/marker	Two-point lod score at different θ values									
	0 0	0 001	0 01	0.05	0.10	0.20	0 30	0.40	Z max	θ
APOA2	∞	-3 162	1 181	0 119	0 617	0 965	0 833	0 432	0 974	0 22
D1S104	0 952	0 953	0 972	1 160	1 272	1 137	0 762	0 304	1 276	0 11
D1S61	7 270	7 258	7 152	6 668	6 034	4 659	3 114	1 393	7 270	0 00
LAMB2	∞	0 006	0 963	1 463	1 503	1 246	0 827	0 356	1 513	0 08
F13B	-∞	-0 812	1 350	2 862	3 152	2 776	1 919	0 829	3 152	0 11

FIG. 3 Identification of the factor V gene muta-
tion in a patient homozygous-deficient in APC
cofactor II. a, Autoradiogram showing the nucleo-
tide substitution in a patient classified as homo-
zygous-deficient in APC cofactor II. Part of the
nucleotide sequence of the non-coding strand of
a cDNA PCR fragment (coding for amino acids
417–572 in human factor V[13]) is shown for one
patient (P) and one non-APC-resistant control (C).
Arrows indicate the location of the 1,691G → A
transition, which predicts the replacement of Arg
506 by Gln. b, Schematic representation of the
factor V molecule. Human factor V is a 330K
glycoprotein which contains several types of
internal repeats[13]. Activation by factor Xa results
in the formation of a 105/220K heterodimer
$(A_1A_2/B'A_3C_1C_2)^{22}$; activation by thrombin results
in the formation of a 105/74K heterodimer
$(A_1A_2/A_3C_1C_2)^{21}$. APC binds to the A3 domain of
factor Va[18,19] and inhibits bovine factor Va by
cleavage in the A2 domain after Arg 505 (ref.
20). The amino-acid sequences surrounding the
(putative) APC cleavage site in human (Arg 506)
and bovine (Arg 505) factor Va[26] are shown. In
the APC-resistant patient, Arg 506 has been
replaced by Gln. c, Resistance of factor Xa-activ-
ated factor V (Q506) to inactivation by APC.
Al(OH)$_3$-adsorbed and fibrinogen-depleted
plasma (for 2 h at 37 °C using 0.3 U ml^{-1}; Arvin) containing either factor
V R506 or factor V Q506 was treated with factor Xa (2 nM) in the
presence of 20 mM CaCl$_2$ and 20 μM PS/PC (25/75). After 8 min, when
the factor Va level had reached a plateau, 1.9 nM APC or buffer was
added. At different time intervals, 10 μl sample was diluted 1/100 in
'stop' buffer (50 mM Tris-HCl, pH 7.9, 180 mM NaCl, 0.5 mg ml^{-1} OVA,
5 mM CaCl$_2$ and 0.5 μg ml^{-1} heparin) and directly assayed for factor Va
activity as described[27]. The factor Va activity measured after complete
activation of 0.70 U ml^{-1} FV (R506) (0.64 μM thrombin min^{-1}) or
0.49 U ml^{-1} FV (Q506) (0.20 μM thrombin min^{-1}) is arbitrarily put at
100%; ○, no APC; ●, +APC.
METHODS. cDNA synthesis: RNA was isolated[28] from the lymphocyte
fraction of 10 ml citrated blood of consenting patients and non-APC-
resistant controls. RNA (1 μg) was used as template for first-strand cDNA
synthesis in the presence of mixed random hexamers using the

superscript kit (BRL). Amplification of cDNA fragments: the primers 5'-
GCATTTACCCTCATGGAGTG-3' (PR-764, nucleotides (nt) 1,421–1,440;
ref. 13) and 5'-CAAGAGTAGTTATGCTCTCAGGCAC-3' (PR-856, nt 1,867–
1,891; ref. 13) amplify the region coding for residues 417–572 which
contains the putative APC cleavage site; the primers 5'-
CACGTGGTTCACTTTCACGG-3' (PR-849, nt 5,608–5,627; ref. 13) and 5'-
TGTGGTATAGCAGGACTTCAGGTA-3' (PR-848, nt 6,040–6,063; ref. 13)
amplify the region coding for amino-acid residues 1,812–1,963, which
contains the APC-binding region. PCR conditions are described in Fig.
4 legend. PCR fragments were purified on ultra-low-gelling temperature
agarose and directly sequenced as before[29] using the same primers
as in the PCR reaction. One additional primer was synthesized to aid
sequencing of the APC-binding region: 5'-TATAAGATCCACCATTGT-3' (PR-
847, nt, 5,905–5,927; ref. 13).

FIG. 4 Association of APC resistance with the presence of
a 1,691A allele of factor V. a, Cosegregatior of 1,691A with
APC resistance. Upper, position of the individuals in the
pedigree shown in Fig. 2a and their n-APC-SR, if available
(II6 was on oral anticoagulant treatment). Middle, MnII
digestion of the 267-bp PCR fragment. Lower, dot-blot
hybridization of the 222-bp fragment with the biotinylated
oligonucleotide specific for the 1,691A allele (PR-1005). b,
Dot-blot hybridization of the 222-bp PCR fragments of 64
thrombosis patients with n-APC-SRC < 0.84 and of their 64
matched controls with the biotinylated oligonucleotide
specific for the 1,691A allele (PR-1005). All patients (P) and
controls (C) gave their informed consent. Slashes denote
positions of failed PCR reactions in this experiment.
METHODS. Amplification of genomic fragments containing
1,691G/A. For MnII digestion a 267-bp fragment was
amplified using as 5' primer 5'-TGCCCAGTGCTTAACAAG-
ACCA-3' (PR-6967; nt 1,581–1,602; ref. 13) and as 3'
primer 5'-TGTTATCACACTGGTGCTAA-3' (PR-990; nt 127 to
−146 in intron 10). For dot-blot hybridization, a 222-bp
fragment was amplified using as 5' primer 5'-GAGAGAC-
ATCGCCTCTGGGCTA-3' (PR-6966, nt 1,626–1,647; ref. 13)
and as 3' primer PR-990. Conditions: 125 μl of a mixture
containing 54 mM Tris-HCl, pH 8.8, 5.4 mM MgCl$_2$, 5.4 μM
EDTA, 13.3 mM (NH$_4$)$_2$SO$_4$, 8% DMSO, 8 mM β-mercapto-
ethanol, 0.4 mg ml^{-1} BSA, 0.8 mM of each nucleoside tri-
phosphate, 400 ng of each primer, 200–500 ng DNA and 2 U Taq
polymerase were subjected to 36 cycles of 91 °C (40 s), 55 °C (40 s)
and 71 °C (2 min). The 267-bp fragment (7–10 μl) was digested with
0.4 U MnII (Biolabs): the 1,691G fragment will give fragments of 67, 37
and 163 bp, whereas the 1,691A fragment will give fragments of 67
and 200 bp. The 222-bp fragment (~100 ng) was used for dot-blot

hybridization with biotinylated sequence-specific oligonucleotides (5'-
TGGACAGGCgAGGAATAC-3' (PR-1006; nt 1,682–1,699; ref. 13) for
detection of 1,691G and 5'-TGGACAGGCaAGGAATAC-3' (PR 1005) for
detection of 1,691A. Procedures have been described[30]. After hybrid-
ization, stringency washing with PR-1006 was at 53 °C, and with PR-
1005 at 52 °C.

plete cosegregation of heterozygosity for the 1,691G → A mutation with APC resistance (n-APC-SR < 0.84) as shown for part of the pedigree (Fig. 4a). Four patients (II.6, II.8, II.14, III.22), for whom no n-APC-SR could be determined because of oral anticoagulant treatment, were found to be heterozygous.

In a previous study of 301 consecutive patients who had suffered a first episode of deep vein thrombosis and of 301 age- and sex-matched controls from the general population, 64 APC-resistant thrombosis patients had been identified[5]. These 64 patients and their 64 controls were screened for the presence of the G → A substitution. Seventy had n-APC-SRC <0.84 (64 patients, 6 controls), of which 56 carried the mutation (53 patients, 3 controls), in both alleles in six of the patients (mean n-APC-SR, 0.43; range, 0.41–0.44) and in one allele in 50 patients (mean n-APC-SR, 0.57; range, 0.50–0.67). The remaining 14 APC-resistant individuals did not carry the mutation and had only a marginally reduced n-APC-SR (mean n-APC-SR, 0.78; range, 0.70–0.83). None of the 58 individuals who were not APC-resistant carried the mutation (mean n-APC-SR, 0.99; range, 0.83–1.19). Further, none of 100 consecutive thrombosis patients with n-APC-SR > 0.84 was a carrier of the mutation, whereas 3 of their 100 matched controls were, as expected. These three (n-APC-SR values 0.57, 0.58 and 0.59) were the only controls with n-APC-SR < 0.84.

Our results show that 80% of the individuals with n-APC-SR < 0.84 and 100% of those with n-APC-SR < 0.70 are heterozygotes or homozygotes for the mutation and that all carriers of the mutation have n-APC-SR < 0.7. The high frequency of the mutated allele in the Dutch population (about 2%) combined with our previous finding[5] that APC resistance is a common and strong risk factor for deep vein thrombosis, makes this hereditary factor V defect the most common hereditary blood coagulation disorder identified so far. A founder effect may be involved in the spread of this disorder in the population, as suggested by the overrepresentation of the common Hinf1 allele of the factor V gene (cytosine at nucleotide 2,298; Fig. 2 legend) in carriers of the factor V Leiden mutation; the frequency of 2,298C was 0.96 in 53 carriers of the mutation and 0.73 in 69 non-carriers ($\chi^2_{diff} = 30.4$, d.f. = 1; $P < 0.001$). ☐

1 Esmon, C T Arteriosclerosis and Thrombosis 12, 135–145 (1992)
2 Walker, F J & Fay P J FASEB J 6, 2561–2567 (1992)
3 Walker, F J J biol Chem 255, 5521–5524 (1980)
4 Dahlback, B , Carlsson, M & Svensson, P J Proc natn Acad Sci USA 90, 1004–1008 (1993)
5 Koster, T et al Lancet 342, 1503–1506 (1993)
6 Svensson, P J & Dahlback, B New Engl J Med 300, 517–522 (1994)
7 Griffin, J H , Evatt, B , Wideman, C & Fernandez J A Blood 82, 1989–1993 (1993)
8 Allaart, C F et al Lancet 341, 134–138 (1993)
9 Engesser, L , Broekmans, A W , Briet, E , Brommer E J P & Bertina, R M Ann int Med 106, 677–682 (1987)
10 Hirsh, J , Piovella, F & Pini, M Am J Med 87, 345–385 (1989)
11 Dahlback, B & Hildebrand, B Proc natn Acad Sci USA 91, 1396–1400 (1994)
12 Wang, H , Riddell, D C Quinto, E R , MacGillivray R T A & Hamerton, J L Genomics 2, 324–328 (1988)
13 Jenny, R J et al Proc natn Acad Sci USA 84, 4846–4850 (1987)
14 Kane, W H & Davie, E W Proc natn Acad Sci USA 83, 6800–6804 (1986)
15 Kane, W H , Ichinose, A , Hagen, F S & Davie, E W Biochemistry 26, 6508–6514 (1987)
16 Cripe, L D , Moor, K D & Kane, W H Biochemistry 31, 3777–3785 (1992)
17 Shen, N L L et al J Immun 150, 2992–3001 (1993)
18 Walker, F J , Scandella, D & Fay, P J J biol Chem 265, 1484–1489 (1990)
19 Walker, F J & Fay, P J J biol Chem 265, 1834–1836 (1990)
20 Odegaard, B & Mann, K J biol Chem 262, 11233–11238 (1987)
21 Suzuki, K , Dahlback, B & Stenflo, J J biol Chem 257, 6556–6564 (1982)
22 Monkovic, D D & Tracey, P B Biochemistry 29, 1118–1128 (1990)
23 Yang X J et al Biochem J 272, 399–406 (1990)
24 Triglia, T , Peterson, M G & Kemp D J Nucleic Acids Res 16, 8186 (1988)
25 NIH/CEPH Collaborative Mapping Group Science 258, 67–86 (1992)
26 Guinto E R , Esmon C T , Mann K G & MacGillivray R T A J biol Chem 267, 2971–2978 (1992)
27 Pieters, J & Lindhout T Blood 72, 2048–2052 (1988)
28 Chromczynski, P & Sacchi, N Analyt Biochem 162, 156–159 (1987)
29 Reitsma, P H , Poort, S R , Allaart C F Briet, E & Bertina, R M Blood 78, 890–984 (1991)
30 Verduyn W et al Hum Immun 37, 59–67 (1993)