Factor XSanto Domingo. Evidence that the severe clinical phenotype arises from a mutation blocking secretion.

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Research Article

Factor X (FX) is a vitamin K-dependent plasma protein required for the intrinsic and extrinsic pathways of blood coagulation. FXSanto Domingo is a hereditary FX deficiency which is characterized clinically by a severe bleeding diathesis. The proposita has a FX activity of less than 1% and a FX antigen of less than 5%. We have determined the molecular basis of the defect in the FXSanto Domingo gene by amplification of all eight exons with polymerase chain reaction and subsequent sequence analysis. The patient is homozygous for a G----A transition in exon I at codon -20 (numbering the alanine at the NH2 terminus of the mature protein as +1), resulting in the substitution of arginine for glycine in the carboxy-terminal part of the signal peptide. This amino acid change occurs near the presumed cleavage site of the signal peptidase. We hypothesized that the mutation might prevent cleavage by the signal peptidase which in turn would impair proper secretion of the FX protein. To test this hypothesis, we compared the expression of wild type and mutant FX cDNA in a human kidney cell line. Wild type and mutant constructs in the expression vector pCMV4 were introduced into the human embryonic kidney cell line 293 by calcium phosphate transfection. FX antigen levels in the supernatant of the cells harboring the wild type construct […]

Factor $X_{Santo\ Domingo}$

Evidence that the Severe Clinical Phenotype Arises from a Mutation Blocking Secretion

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Abstract

Factor X (FX) is a vitamin K–dependent plasma protein required for the intrinsic and extrinsic pathways of blood coagulation. $FX_{Santo\ Domingo}$ is a hereditary FX deficiency which is characterized clinically by a severe bleeding diathesis. The propositas has a FX activity of < 1% and a FX antigen of < 5%. We have determined the molecular basis of the defect in the $FX_{Santo\ Domingo}$ gene by amplification of all eight exons with polymerase chain reaction and subsequent sequence analysis. The patient is homozygous for a G → A transition in exon I at codon −20 (numbering the alanine at the NH_2 terminus of the mature protein as +1), resulting in the substitution of arginine for glycine in the carboxy-terminal part of the signal peptide. This amino acid change occurs near the presumed cleavage site of the signal peptidase. We hypothesized that the mutation might prevent cleavage by the signal peptidase which in turn would impair proper secretion of the FX protein. To test this hypothesis, we compared the expression of wild type and mutant FX cDNA in a human kidney cell line. Wild type and mutant constructs in the expression vector pCMV4 were introduced into the human embryonic kidney cell line 293 by calcium phosphate transfection. FX antigen levels in the supernatant of the cells harboring the wild type construct were 2.4 $\mu g/10^7$ cells per 24 h, whereas antigen levels in media from cells containing the $FX_{Santo\ Domingo}$ construct were undetectable. No FX antigen was detected in the cell lysates of cells transfected with the mutant construct. To insure that the difference in protein levels was not due to a difference in steady state levels of mRNA, Northern analysis was performed on RNA from the cell lysates of both constructs. The results showed a transcript of the same size, present in roughly equal amounts, in both cases. Thus, the defect in the signal sequence of $FX_{Santo\ Domingo}$ exerts its effect posttranscriptionally. $FX_{Santo\ Domingo}$ is the first described example of a bleeding diathesis due to a mutation in the signal sequence. (*J. Clin. Invest.* 1991. 88:1685–1689.) Key words: factor X • signal sequence • bleeding diathesis • factor X deficiency

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Introduction

Factor X (FX)[1] is a vitamin K–dependent plasma glycoprotein required for blood coagulation (1). It is synthesized in hepatocytes as a single polypeptide chain, or prepro-FX, and undergoes several modifications before it is constitutively secreted into plasma. In a cotranslational event, the signal sequence is cleaved off from the NH_2-terminal portion of the primary translational product of FX. Further posttranslational steps include γ-carboxylation of 11 glutamic acid residues located near the NH_2 terminus, cleavage of the propeptide, glycosylation, and beta hydroxylation. The amino acid sequence of FX has been derived from FX cDNA's and direct amino acid analysis (2–5). The gene has also been isolated and partially characterized. It has been mapped to chromosome 13q32-qter (6), where it spans ~ 25 kb; it consists of seven introns and eight exons and has structural homology with the genes encoding the other vitamin K–dependent clotting factors (7). The functional domains of the protein are represented by different exons: exon I codes for the signal sequence, exon II for the propeptide, and the γ-carboxyglutamic acid-rich domain, exon III for the short aromatic acid-rich stack, exons IV and V for the epidermal growth factor–like domains, exon VI for the activation peptide, and exons VII and VIII for the catalytic domain (7).

Congenital FX deficiency is inherited as an autosomal recessive trait (8). Considerable phenotypic heterogeneity exists among FX variants (9–11). The molecular basis of congenital FX deficiency has been completely elucidated in only three families so far. Two of these are homozygous for defects in exon II (12) and exon VIII (13), respectively. One compound heterozygote has been reported (14). Here we report another homozygous FX deficiency ($FX_{Santo\ Domingo}$) in which a point mutation in exon I leads to a severe bleeding disorder in the affected proposita.

Methods

Patient data. The proposita is a 16-yr-old female from Santo Domingo, Dominican Republic, who came to medical attention because of heavy menstrual bleeding at menarche. This was associated with significant blood loss, requiring transfusion, and on occasion, treatment with prothrombin complex concentrates. Blood samples were obtained from the proposita and her parents after informed consent. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were performed using standard methods. FX antigen levels were determined by immunoassay using a FX-specific sandwich ELISA. Four different monoclonal antibodies were used as capture antibodies (gift of Dr. William Church, University of Vermont, and Dr. D. M. Monroe, University of North Carolina). One of these has been extensively characterized and recognizes an epitope in the heavy chain (15); the other three also recognize epitopes in the heavy chain. Plates are coated first with

1. *Abbreviations used in this paper:* aPTT, activated partial thromboplastin time; FX, Factor X; PT, prothrombin; TBE, Tris-borate-EDTA buffer.

the monoclonal antibody; the second antibody is a rabbit polyclonal anti-human FX (12). Factor X activity levels were determined using a one-stage clotting assay.

DNA analysis. Genomic DNA was prepared from the patients' peripheral blood using standard methods (16), and Southern blots were performed to determine whether the gene was grossly intact. 10 μg of genomic DNA from the proposita and the same amount from a nonrelated normal control were digested with 30 U of Eco RI at 37°C for 3 h. The digest was electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane according to the method of Southern (17). A full-length human FX cDNA (gift of Dr. D. Stafford) was radiolabelled with ^{32}P dCTP by random priming using the manufacturer's conditions (Boehringer-Mannheim Biochemicals, Indianapolis, IN). 20 ng of labelled probe with a sp act of 1.8×10^9 cpm/μg was added to the filter incubating in 6× SSC, 0.01 M EDTA, 0.1 M $K_2H_2PO_4$, 5× Denhardt's solution, 0.5% SDS, and 250 μg/ml salmon sperm DNA. After incubation for 12 h at 68°C the filter was washed in 2× SSC, 0.5% SDS twice for 15 min at room temperature, in 0.1× SSC, 0.5% SDS for 45 min at 68°C, and exposed to x-ray film at −70°C for 24 h.

All eight exons of FX were isolated using the enzymatic amplification technique (18). Synthetic oligonucleotides derived from intron sequences flanking the 5′ and 3′ ends of each exon were used to prime amplification (12). Hind III restriction sites were built in at the 5′ ends of each oligonucleotide. Target sequences were amplified in a 100-μl volume containing 1 μg of genomic DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.2 μM of each deoxyribonucleotide (dNTP:dCTP, dGTP, dATP, dTTP), 1 μM of each primer, and 1 U Taq polymerase. The samples were overlaid with 100 μl mineral oil to prevent evaporation and subjected to 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and primer extension at 72°C for 3 min on a DNA Thermal Cycler R (Perkin-Elmer Cetus, Emeryville, CA). The amplified DNA was electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer (TBE) and the amplified region cut out from the gel. The recovered gel slice was crushed and incubated with 50 μl TBE, 50 μl phenol, and 50 μl chloroform:isoamyl alcohol (24:1) for 15 min at −70°C. After spinning for 15 min in a microfuge the supernatant was recovered and the DNA precipitated with ethanol. After digestion with Hind III the fragments were subcloned into M13 sequencing vectors and sequenced as previously described (19).

Exon I was also amplified as described above to perform restriction analysis with Dde I. 30 base synthetic oligonucleotides derived from the 5′ untranslated region and from intron A were used in this amplification to yield a DNA fragment of 213 bp containing exon I and a single naturally occurring Dde I site. DNA fragments were electrophoresed and purified as described above. Digestions were carried out according to the manufacturer's conditions with Dde I (New England Biolabs, Beverly, MA). Digested DNA was electrophoresed on a 6% agarose gel (Nu Sieve GTG; FMC Corp., FMC BioProducts, Rockland, ME) in TBE buffer. Exon I amplified from normal DNA was used as a control in all restriction digests.

In vitro expression. A partial human FX cDNA which contains the complete FX coding sequence, a translation initiation sequence, and part of the 3′ untranslated region was inserted in the multiple cloning site of the phagemid pMA254. The point mutation found in exon I of the $FX_{Santo\ Domingo}$ gene was introduced into the pMA254-FX plasmid by oligonucleotide hybridization (20) and the gapped duplex method. The presence of the point mutation in the mutagenized plasmid was confirmed by DNA sequence analysis. The plasmid pCMV4 was used as the expression vector (21). The normal FX cDNA was inserted in the multiple cloning site of this plasmid in order to express the wild type FX. The expression plasmid of $FX_{Santo\ Domingo}$ was constructed by deletion of a Sma I-Sac II DNA fragment from the normal FXcDNA in pCMV4. This fragment includes the nucleotide that is mutated in $FX_{Santo\ Domingo}$. The deleted fragment was replaced by the corresponding fragment of pMA254 that carries the point mutation of the $FX_{Santo\ Domingo}$ genotype. The correct insertion of the fragment and the presence of the mutation were again confirmed by sequence analysis.

The constructs were introduced into the human embryonic kidney cell line 293 by calcium phosphate transfection (22). All transfections were done in duplicate. A mock transfection (no DNA) served as a control. Cells were grown in DMEM/F12 medium in the presence of 10% FCS. 24 h before collecting samples, culture medium was changed and fetal calf serum was omitted to eliminate any possibility of antibody cross-reactivity with bovine FX. The supernatant was collected 72 h after transfection and analyzed for the presence of FX antigen, using the immunoassay described above. The transfected cells were also collected, lysed, and assayed for the presence of FX. For the cell lysis procedure, confluent cells in a 10-cm dish were washed twice with PBS, then covered with buffer containing 0.5% NP-40, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol. The cells were scraped from the plate and centrifuged at 12,000 *g* for 15 min at 4°C. FX antigen levels in the supernatant were determined. Total RNA was isolated from the transfected cells by the guanidinum-phenol-chloroform method (23). 10 μg of total RNA was loaded on a formaldehyde-agarose (1%) gel and electrophoresed for 2 h at 75 V. RNA was transferred onto a nitrocellulose filter, probed with a radiolabelled FX cDNA, and exposed to x-ray film at −70°C for 12 h.

Results

Clinical data and gene analysis. Results of coagulation studies are described in Table I. The proposita has a markedly prolonged PT and aPTT. Factor assays revealed a FX activity of < 1%; the activities of all other coagulation factors were within normal limits. The parents both had FX activity levels in an intermediate range, 29–38%. The FX antigen levels were 5% of normal in the proposita and 50% of normal in the parents (Table I). Southern blot analysis of the proposita's EcoRI digested DNA probed with an FX cDNA showed the same pattern of bands as normal DNA (data not shown). Thus, there is no evidence of a gross gene deletion, insertion or rearrangement.

Sequence analysis of the coding region and the exon-intron junctions of the proposita's FX gene revealed a single base mismatch when compared with the normal sequence. A G → A point mutation at the first nucleotide of codon −20 results in the substitution of an arginine (AGG) for a glycine (GGG) (Fig. 1). The mutation creates a new Dde I restriction site within exon I (Fig. 1). Enzymatically amplified exon I from a mutant allele therefore shows one additional Dde I site which can be used for discrimination between a normal, a homozygous, and a heterozygous genotype with respect to the mutation in exon I. Dde I restriction of DNA amplified from exon I of a normal control showed DNA fragments of 113 bp and 100 bp due to the naturally occurring Dde I site (Fig. 2). In the DNA amplified from exon I of the proposita, the 100-bp fragment was further cleaved into fragments of 70 bp and 30 bp due to the

Table I. Factor X Coagulation Values for Normal Control and Santo Domingo Kindred

	PT	aPTT	Antigen	Activity
	s	*s*		
Normal	9–12.5	22–35	10 μg/ml	60–150%
Father	11.7	28.7	5 μg/ml	38%
Mother	12.4	32.6	5 μg/ml	29%
Patient	29.8	74.6	0.5 μg/ml	<1%

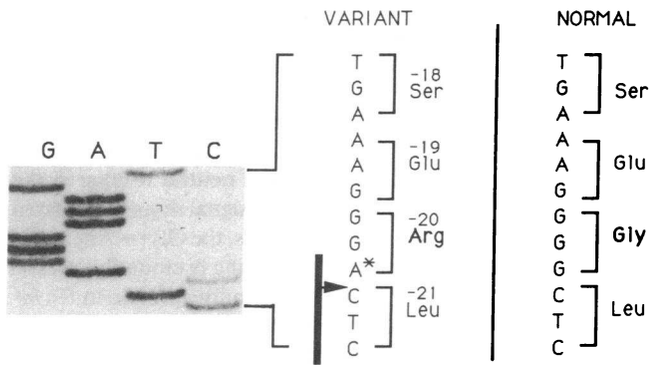

Figure 1. Sequence analysis of exon I of $FX_{Santo\ Domingo}$. The G → A transition at the first nucleotide of codon −20 changes glycine to arginine. The recognition site for the restriction enzyme Dde I CTNAG, where N is any nucleotide, is indicated by the bar. It is present in FX_{SD} but not in wild type.

additional Dde I site created by the mutation. She is therefore homozygous for the defect in exon I. Exon I amplified from the parents showed bands at 113 bp, 100 bp, 70 bp, and 30 bp, indicating a heterozygous genotype.

In vitro expression system. To test the effect of the point mutation on FX expression we expressed a normal FX cDNA and a FX cDNA carrying the "Santo Domingo" mutation in a transient expression system (Table II). The wild type FX construct yielded a FX antigen concentration in the cell supernatant of 300 ng/ml per 24 h. Normalized to the number of cells, this concentration represents 2.4 μg FX antigen/10^7 cells per 24 h. In contrast, no FX antigen was detectable in the supernatant of the cells that were transfected with the $FX_{Santo\ Domingo}$ construct. The lower limit of detection of the ELISA is 10 ng/ml. When the transfected cells were lysed and assayed for the presence of FX, no FX protein was detectable in the cells transfected with the Santo Domingo construct. FX was present in the cells transfected with the wild type construct at a concentration of 300 ng/ml. Northern blot analysis of cell lysates showed a FX mRNA of the same size, present in roughly equal amounts, in both the wild type and the Santo Domingo transfectants (Fig. 3).

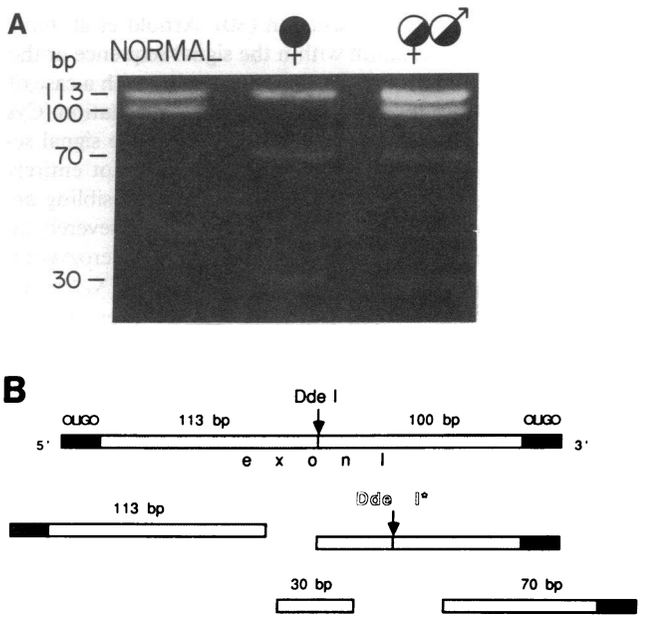

Figure 2. (*A*) Restriction digests of amplified exon I from the proposita and her parents. Dde I restriction digest of amplified exon I from a normal control, yielding fragments of 113 and 100 bp, is shown on the left. In the center lane, the digest from the proposita shows an absence of the 100-bp band, and its replacement by the predicted 70- and 30-bp fragments. Similar analysis on the father of the proposita discloses heterozygosity, with the 100 bp, and the 70- and 30-bp fragments, all present. Analysis of the mother yielded the same results (not shown). (*B*) Restriction map of amplified exon I. Priming oligomers (ref. 12) are marked by the crosshatched boxes. The single naturally occurring Dde I site divides the exon into 113- and 100-bp fragments. The Santo Domingo mutation creates an additional Dde I site that divides the 100-bp fragment into 70- and 30-bp fragments.

Discussion

Analysis of the gene coding for $FX_{Santo\ Domingo}$ disclosed that the FX deficiency in this patient is associated with a single point mutation in exon I, which encodes the signal sequence. The proposita is homozygous for the defect, and is severely affected, with a FX activity of < 1% and FX antigen of only 5%. Her parents are heterozygous for the defect and thus, in accordance with the autosomal recessive character of FX deficiency, asymptomatic. $FX_{Santo\ Domingo}$ is the fourth FX deficiency that has been completely elucidated at the molecular level (12–14). It is the first mutation in a FX deficiency, however, in which the causative effect of the mutation has been proved by expression in vitro of the mutant cDNA and subsequent analysis of the expressed phenotype. The results of our expression experiments in vitro parallel the situation in vivo, as the lack of FX expression in vitro correlates well with the low FX antigen levels in the plasma of the proposita. We therefore conclude that the point mutation in exon I and the resulting substitution of arginine for glycine at position −20 (numbering the alanine at the NH_2 terminus of the mature protein as +1) cause the $FX_{Santo\ Domingo}$ phenotype.

Amino acid −20 is part of the prepro region of the FX protein. The signal sequence serves the function of targeting the elongating polypeptide chain to the site of translocation into the endoplasmic reticulum, where a number of critical posttranslational steps occur. The signal sequence is cleaved from the protein in a cotranslational event (24) as the newly synthesized protein is translocated into the lumen of the endoplasmic reticulum. The propeptide, which is still attached and serves as a recognition site for the γ-carboxylase (25), is cleaved in a subsequent posttranslational modification. While the NH_2 terminus of the mature protein unambiguously indicates where the propeptide is cleaved, the cleavage site of the signal se-

Table II. Factor X ELISA Results in Transfectants

	Supernatant	Cell lysate
Mock	ND	ND
FX_{wt}	2.4 μg/10^7 cells per 24 h	300 ng/ml
FX_{SD}	ND	ND

ND, not detectable. All transfections done in duplicate.

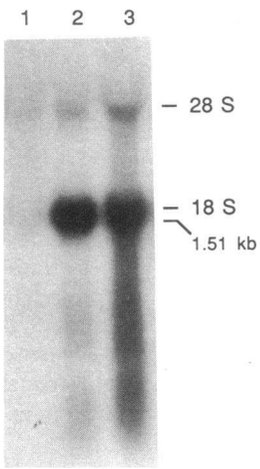

Figure 3. Northern analysis of total RNA from transfected cells. 10 μg total RNA from mock transfected (lane *1*), wild type (lane *2*), and FX_{SD}-transfected cells (lane *3*) were loaded on a gel and transferred to nitrocellulose. The blot was hybridized against radiolabelled human FX cDNA. The 1.51-kb FX transcript is present in the wild type and FX_{SD} transfectants.

quence in the human prepro FX has not been established with certainty.

Several lines of evidence suggest that the signal peptidase cleavage site is located between Ser(−18) and Leu(−17). First, organization of the FX gene follows a structure-function pattern whereby the coding regions for the various functional domains of the FX protein are found in different exons (7). Exon I encodes the signal peptide domain of all vitamin K–dependent coagulation factors. Exon I in the FX gene codes for amino acids Met(−40) through Leu(−18). Thus, exon I exactly encodes the complete signal sequence assuming the signal peptidase cleavage site is COOH-terminal to Leu(−18). Second, the COOH termini of signal sequences are characterized by small, neutral amino acid residues at positions −1 and −3 (numbering the very COOH-terminal amino acid of the signal sequence as −1) (26). The conservation of small neutral amino acids at these residues is sometimes referred to as the −1, −3 rule. In aligning various signal sequences, von Heijne was able to empirically develop rules that allow prediction of the actual cleavage site of the signal sequence by signal peptidase (26). Application of these rules to FX predicts a highly favorable cleavage site for the signal peptidase between residues −18 and −17 (Fig. 4). Finally, presumption of cleavage at this point is further supported by the fact that the bovine FX signal sequence, which is

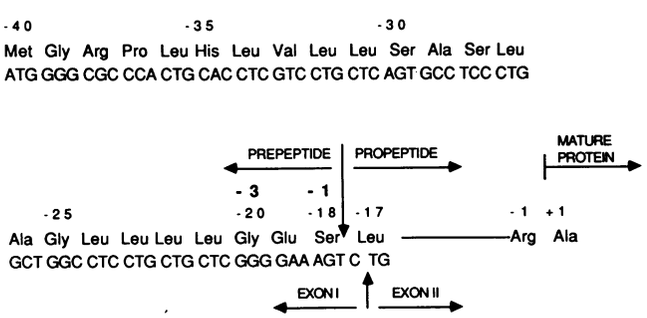

Figure 4. The prepropeptide of human Factor X. The first 40 residues of the translation product are absent from the mature protein that circulates in plasma. These residues are generally numbered −1 to −40, proceeding back from the start site of the mature protein. The cleavage site for the prepeptide is most likely at −17/−18, so that −18 corresponds to residue −1 in signal peptide nomenclature. Similarly, the site of substitution in FX_{SD} is −3.

very similar to the human FX signal sequence, has been reported to be cleaved exactly at this point (27).

The substitution of Arg for Gly, occurring at residue −20 numbering from the NH_2 terminus of the mature protein, falls at the critical −3 residue, numbering from the presumed COOH terminus of the signal sequence. Consistent with the −1, −3 rule, amino acid −3 is a small neutral residue in the majority of prokaryotic and eukaryotic signal sequences. Based on data generated in prokaryotic systems, the Gly → Arg mutation at −3 is predicted to impair processing profoundly. An Ala → Arg mutation at −3 of the maltose binding protein in *Escherichia coli* results in extremely inefficient processing, with only ~ 7% of premaltose binding protein being cleaved to mature product (28). More conservative substitutions, e.g., glycine, valine, leucine, had no effect on processing. One possibility then is that the substitution introduced in FX_{SD} by Arg-3 may interfere with processing (cleavage by signal peptidase) in a similar manner.

There are few data regarding naturally occurring substitutions in human signal sequences, and the substitutions previously reported do not occur at the carboxy terminus of the signal peptide. Albumin Redhill (29) is characterized by an Arg → Cys substitution within the propeptide, which appears to create an alternate cleavage site for signal peptidase. The resulting albumin variant contains a single extra NH_2-terminal amino acid but is not associated with any disease state. Similarly, a 9-bp insertion/deletion within the hydrophobic core of the signal peptide of human apolipoprotein B has been reported, but this appears to be a benign polymorphism with no influence on signal peptide function (30). Arnold et al. have recently reported a mutation within the signal sequence of the preproparathyroid hormone gene, in association with a case of familial isolated hypoparathyroidism (31). The mutation (Cys → Arg) occurs within the hydrophobic core of the signal sequence. Interpretation of the data in this case is not entirely straightforward, since the patient and an affected sibling are heterozygous for the signal peptide mutation and severely affected, while the father of these two patients, also heterozygous, is not clinically affected. In contrast, the case of FX_{SD} is less ambiguous, since the parents are both heterozygous for the mutation, have intermediate FX activity levels, and are clinically unaffected, whereas the proband, homozygous for the mutation, has a FX activity level of < 1%, and is severely affected clinically.

To determine whether the signal peptide mutation gives rise to the observed phenotype, constructs bearing wild type Factor X and FX_{SD} were transfected into a human embryonic kidney cell line. Such a eukaryotic expression system allows one to determine the functional consequences of the mutation on cellular secretion. While the wild type was normally expressed as expected, no detectable FX antigen was present in the media of the cells harboring the FX_{SD} construct. The nature of the antibodies used in the ELISAs, one polyclonal, the other a monoclonal directed against an epitope in the catalytic domain, makes it unlikely that an aberrantly processed FX, with an additional NH_2-terminal sequence, was present but not recognized. Differences in expression of the two constructs at the level of transcription were ruled out by Northern analysis, which demonstrates equivalent steady-state levels of FX mRNA (Fig. 3). Analysis of cell lysates from the transfected cells showed that FX antigen was present in cells transfected with the wild type construct, but absent in those harboring the

FX_{SD} construct. The precise mechanism of the profound impairment of expression is unclear. Future studies using in vitro systems will be directed toward determining which of several possible mechanisms, including failure of translation, of targeting, or of signal sequence cleavage, accounts for the impaired expression.

In summary, we have determined the causative mutation in a case of severe Factor X deficiency. The proposita is homozygous for a G → A transition that results in a glycine → arginine substitution within the signal peptide. Restriction analysis demonstrates that the parents are heterozygous for the same mutation; their FX activity levels are 30–40% range, and the proposita's is < 1%. Expression of the mutation in a mammalian expression system demonstrates that, in contrast to wild type FX, Factor X_{SD} is not secreted into the media or detectable in the cell lysates. Thus, the signal sequence mutation accounts for the observed phenotype. Among the three previously described mutations causing Factor X deficiency, and among > 200 mutations causing F.IX deficiency, there have been no examples of signal peptide mutations causing a bleeding diathesis (12–14, 32). Further study of this mutation may yield additional insights into the mechanism of action of signal sequences.

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