A 5.3-kb Deletion Including Exon XIII of the Protein S α Gene Occurs in Two Protein S-Deficient Families


Genomic DNA samples from 12 protein S-deficient families with hereditary thrombophilia were analyzed by Southern hybridization using protein S cDNA probes. Protein S-deficient members of families A and B possessed identical restriction fragment length polymorphisms, which suggest the absence of 5.3 kb from one of their protein S α alleles. The abnormal alleles from individuals A7 and B1 were amplified by the polymerase chain reaction using a forward primer in intron K and a reverse primer in exon XIV. The amplified DNA was cloned and sequenced. Sequence comparison with the normal protein S α gene showed that most of intron L (roughly 4.7 kb), the entire exon XIII (151 bp), and about a quarter of intron M (407 bp) were missing from both the A7 and B1 clones. Exon XIII contains all three potential N-glycosylation sites in human protein S. This deletion may result in RNA transcripts in which exon XII is spliced to exon XIV. Such an arrangement would generate a stop codon at position 463 and consequently produce a nonglycosylated protein S molecule truncated by 173 amino acids.

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Human protein S genomic clones. Human protein S genomic clones isolated from a xEMBL3 library have been previously characterized by restriction mapping and DNA sequencing. Composite maps of the 3′-most 21 kb of the Psa gene and the corresponding region of the Psb gene are shown in Fig 1.

Protein S-deficient families. Initially, DNA samples from the probands of 12 protein S-deficient families along with DNA from four normal volunteers were analyzed by Southern hybridization. Two probands exhibited identical restriction fragment length polymorphisms (RFLPs), and consequently DNA from their relatives was analyzed. Family A (Fig 2), which has both protein S- and protein C-deficient members, was characterized by Broxson et al. The clinical data for family B were reported by Comp et al. All protein S-deficient patients examined from these two families had about half the normal level of total protein S antigen, most of which was bound to C4BP (Table 1).

Southern hybridization. Genomic DNA was isolated from peripheral blood by the method of Kunkel et al with modifications described by Miller and Dykes. Ten-microgram samples were digested with a series of restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD) under conditions recommended by the supplier. The DNA fragments were separated by electrophoresis through 0.8% agarose gels at 40 V for about 18 hours. The DNA was then transferred to NitroPlus 2000 membranes (Micron Separations Inc, Westborough, MA) by the method of Smith and Summers. After baking at 80°C for 2 hours, the membranes were prehybridized at 65°C for at least 8 hours in 6× SSC (0.9 mol/L NaCl, 90 mmol/L sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, 10X Denhardt's solution (2% Ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin [BSA]), and 0.1 mg/mL denatured sheared salmon sperm DNA. Protein S cDNA fragments spanning exons X through XV (Fig 1) were isolated from plasmid pHHSIIa and 3′-radiolabeled using the random hexamer primer method of Feinberg and Vogelstein. Unincorporated nucleotides were removed by aqueous ethanol precipitation.

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moved with P-60 columns (BioRad, Richmond, CA). The membranes were hybridized with the labeled cDNA probes at 65°C overnight, then rinsed in 1X SSC/0.1% SDS briefly at room temperature, followed by a 30-minute rinse at 60°C. The rinsed membranes were exposed to XAR5 film (Eastman Kodak Co, Rochester, NY) at -70°C with the aid of an intensifying screen for several days.

**Polymerase chain reaction (PCR).** One-microliter samples of DNA from a normal (A3) and two protein S-deficient individuals (A7 and B1) were amplified using PCR. Primers were synthesized on an Applied Biosystems Inc Model 381 DNA Synthesizer (Foster City, CA) and purified via oligonucleotide purification cartridges. The PCR reactions were performed in 100-μL volumes containing: 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.01% gelatin, 0.2 mmol/L each dNTP (dATP, dCTP, dGTP, and dTTP), 1 pmol/L both primers, and 2.5 U Taq polymerase (92°C, 1 minute), annealing (58°C, 1 minute), and extension (72°C, 1.6 minutes). The PCR products were digested with PstI, size-fractionated by electrophoresis through 1.5% low-melt agarose gels (FMC BioProducts, Rockland, ME), and visualized with ethidium bromide.

**Southern blot analysis.** Purified protein S from normal pooled plasma along with plasma from a protein S-deficient and a normal individual of family A were electrophoresed in a nonreducing 12% SDS-polyacrylamide gel (Hoefer Mighty-Small, San Francisco, CA). Proteins were transferred to nitrocellulose by the method of Towbin et al. The nitrocellulose was then blocked with 2% BSA in 0.02 mol/L Tris-Cl pH 7.6, 1.5 mol/L NaCl, 0.05% Tween-20 (T-TBS), and blotted with rabbit antiserum against protein S at a dilution of 1:60,000 in T-TBS containing 1% BSA. The blotted membrane was visualized by incubating with goat antirabbit IgG-biotin conjugate, followed by streptavidin alkaline phosphatase, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

**RESULTS**

Southern hybridization of PstI digested DNA from five protein S-deficient family A members showed a variant 5.3-kb band (Fig 2). In addition, densimetric scanning of this blot also showed that the 10.6-kb band from protein S-deficient individuals was half as intense as that from normal individuals. This PstI RFLP is linked to protein S deficiency in family A through three generations. It was also observed with B1 DNA, although not with DNA from the probands of 10 other protein S-deficient families. This RFLP could be explained by a 5.3-kb deletion between the two PstI sites of the PSa gene that are normally situated 10.6 kb from each other. Note that the PSb does not contribute any hybridizing 10.6-kb PstI fragments.

To further substantiate this hypothesis, DNA samples from families A and B were digested with seven other sets of restriction endonucleases and hybridized with protein S cDNA probes. Two of the resulting autoradiograms are shown in Fig 3. HindIII digestion produced a variant 8.1-kb band and a decrease in the intensity of the normal 13.4-kb band, while EcoRI/BamHI digestion generated a variant 4.3-kb band and a less intense 5.8 kb band. These patterns are consistent with the interpretation that a region of approximately 5.3 kb has been deleted. One would predict
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Fig 2. PstI Southern blot of family A. (A) The family A pedigree with protein S-deficient members shaded. (B) A Southern blot of family A DNA digested with PstI and hybridized with the TaqI-[1777]-EcoRI-[2168] protein S cDNA fragment. Fragment sizes are indicated in kilobases. Normal fragments that correspond to the PSα gene are labeled "E" (expressed gene) and those that correspond to the PSβ gene are labeled "P" (pseudogene). (C) Densitometric tracings of the A3 (normal) and A4 (protein S deficient) lanes in (B).
Table 1. Plasma Protein S Levels for Families A and B

<table>
<thead>
<tr>
<th></th>
<th>Total Protein S (µg/mL)</th>
<th>Free Protein S (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Normal range</td>
<td>16.0-25.4</td>
<td>4.0-10.8</td>
</tr>
<tr>
<td>Family A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>13.7</td>
<td>—</td>
</tr>
<tr>
<td>A3</td>
<td>24.8</td>
<td>8.2</td>
</tr>
<tr>
<td>A4</td>
<td>13.9</td>
<td>—</td>
</tr>
<tr>
<td>A5</td>
<td>8.1</td>
<td>—</td>
</tr>
<tr>
<td>A6</td>
<td>13.7</td>
<td>2.0</td>
</tr>
<tr>
<td>A7</td>
<td>12.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Family B</td>
<td></td>
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</tr>
<tr>
<td>B1</td>
<td>6.2</td>
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<tr>
<td>B2</td>
<td>20.0</td>
<td>7.3</td>
</tr>
<tr>
<td>B3</td>
<td>7.6</td>
<td>0.8</td>
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Total and free protein S levels were determined by Laurell rocket electrophoresis in the case of family A and by enzyme-linked immunosorbent assay for family B. These levels were estimated for warfarin-treated individuals A1, A4, and A5 by calculating the protein S/factor X antigen ratio. Free protein S levels were not determined for these individuals.

The EcoRI/BamHI digestion would result in the fading of not only the 5.8-kb band, but also the 1.4-kb band. However, this distinction was not apparent because the PSβ gene also contributes two 1.4-kb fragments.

A forward primer in intron K and a reverse primer in exon XIV were used to amplify the variant region of A7's and B1's PSα genes (Fig 4). Theoretically, a normal genomic DNA template should produce a 6.8-kb product from both the PSα and PSβ genes upon amplification with this primer pair. However, such a large product is unlikely to be visibly amplified using typical PCR conditions. Thus, only amplification of DNA from the variant allele was anticipated. As shown in Fig 4, amplification resulted in a 2.4-kb product for both A7 and B1 DNA, but not for the normal DNA template (A3). These products were the expected size assuming that 5.3 kb had been deleted from the variant alleles. Smaller products were consistently amplified from all genomic DNA templates tested. The identity of these products is unknown; however, they served as internal controls indicating the presence and amplification of normal DNA template.

The 1.2-kb XbaI/EcoRI fragment from the 2.4-kb B1 PCR product was cloned into pUC-19. This plasmid (pSV1-XE[B1]) along with subclones of it and corresponding RNA splice site. The absence of exon XIII from one PSα allele could generate RNA transcripts, which are spliced in such a way as to juxtapose exons XII and XIV. Because intron L has different splice junction types at its two ends, the joining of exon XII to exon XIV would create a frameshift and alter the subsequent amino acid sequence. In this case, the variant mRNA would contain a stop codon six codons downstream from the 5' end of exon XIV, resulting in a protein truncated by 173 amino acids.

Exon XIII contains all three potential N-glycosylation sites (amino acids 458, 468, and 489) in human protein S. Therefore, the variant form of protein S in deficient members of families A and B would not be expected to contain carbohydrate. A total absence of glycosylation might lead to decreased secretion or increased clearance rates from circulation. Consistent with either of these possibilities is the apparent absence of any significantly truncated forms of protein S in plasma from deficient individuals as assessed by immunoblot analysis (Fig 6). Reduced levels of circulating protein S resulting from either of these possibilities is also consistent with the observed decrease in both total and free protein S in affected family members. The apparent dissociation constant for human protein S/C4BP in plasma has been reported to be 70 nmol/L, which is relatively close to the 300 nmol/L circulating concentration of protein S. Consequently, reduction in the total protein S concentration (with constant C4BP concentration) could significantly increase the proportion of bound protein S, as seen in protein S-deficient family A and B members.

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Fig 3. Southern blots comparing families A and B. DNA samples from a normal (A2) and two protein S-deficient (A1, B1) individuals were digested with the indicated restriction endonucleases and probed with the XbaI(1481)-SalI(1904) protein S cDNA fragment. Fragment sizes are indicated in kilobases.
Fig 4. PCR products from human protein S genomic amplification. Genomic DNA samples from a normal (A3) and two protein S-deficient (A7, B1) individuals were amplified by PCR using a forward primer (5'-TGAAAT-
CCTATACCTATCATTCAGC-
CACTG-3') located in intron K, 45 bp upstream of exon XII, and a reverse primer (5'-ATTGCTT-
TGCCAAGACGGC-3') located within exon XIV, downstream of the SaI site. One fourth of the products were electrophoresed through a 1% agarose gel and stained with ethidium bromide (B). Lane L contains DNA size standards (1-kb Ladder; Bethesda Research Laboratories). Lanes 1, 2, and 3 contain A3, A7, and B1 PCR amplified DNA, respectively. (A) A partial map of the PSa gene spanning exons XI1 through XIV (shaded) and introns K through N. Restriction sites are designated as follows: E, EcoRI; H, HindIII; P, PstI; S, SaI; X, XbaI. The heavy bar beneath the map represents the 2.4-kb region of the normal PSa gene that was amplified from A7 and B1 DNA. The 5.3-kb region missing from the variant alleles is shown with a dashed line.

Fig 5. The composite nucleotide sequence of deletion clones and corresponding regions of normal PSa gene clones. (A) Restriction map of the variant clone pSV1-XE[B1] is drawn above a partial map of the PSa gene. The gene map includes exons XII and XIII (shaded boxes) and introns L and M. Restriction sites are designated as follows: A, Avall; Bg, BglII; D, DraI; E, EcoRI; Hc, HindIII; R, RsaI; X, XbaI. Arrows beneath the maps indicate regions that have been sequenced. A variant clone (pSV1-DHe[A7]) from family A was sequenced in a similar manner. The sequence at the 5' end of both the variant clones was identical to the exon XII/intron L region distinguished with a hatched bar, while the other end matched the region of intron M shown with a striped bar. The composite sequence for both normal and variant clones is presented in (B and C). Exon/intron junctions and key restriction sites are labeled. The underlined sequence, including the entire exon XIII, is absent from the variant clones. The intron L sequence 3' of the first RsaI site (590 bp) has not previously been reported, nor has the 755-bp sequence 5' of the EcoRI site in intron M.
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[Diagram showing genetic sequences and restriction enzyme sites]

**A**

**B**

**C**

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Department of Family Medicine, East Carolina University, School of Medicine, Greenville NC. The rabbit antiprotein S antiserum was provided by Dr William Church (Thrombosis S.C.O.R. Immunology Core Facility, University of Vermont, Burlington). We thank all of the donors for their generous gifts of blood.

REFERENCES
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A 5.3-kb deletion including exon XIII of the protein S alpha gene occurs in two protein S-deficient families [see comments]

DK Schmidel, RM Nelson, EH Jr Broxson, PC Comp, RA Marlar and GL Long