Molecular Basis for Congenital Deficiency of α_2-Plasmin Inhibitor

A Frameshift Mutation Leading to Elongation of the Deduced Amino Acid Sequence

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Abstract

The present study was designed to elucidate the molecular genetic basis of a familial deficiency of α_2-plasmin inhibitor (α_2PI). Southern blot hybridization analysis with human α_2PI cDNA and genomic DNA probes demonstrated no gross deletion or rearrangement of the gene. By sequencing all the coding exons and exon-intron boundaries of the gene of a homozygote, we identified a single cytidine nucleotide insertion in the exon coding for the carboxyl-terminal region. This frameshift mutation leads to an alteration and elongation of the carboxyl-terminal portion of the deduced amino acid sequence. Synthetic oligonucleotide probes confirmed this frameshift mutation in all the affected family members including both heterozygous parents. In a transient expression assay, the α_2PI level in the culture medium of the cells transfected with the mutated α_2PI expression vector was very low and only 4% of that of the cells transfected with the normal vector, although the transcript levels and the cellular contents of α_2PIs did not differ significantly. Elongation of amino acid sequence in the mutant α_2PI was confirmed by an analysis of α_2PI in a transient expression experiment. These data indicate that this mutation is the cause of α_2PI deficiency in this pedigree.

Introduction

α_2-Plasmin inhibitor (α_2PI),[1] also called α_2-antiplasmin, is a plasma glycoprotein with an estimated molecular mass of 67 kD that contains ~ 11% carbohydrate (1). Its concentration in normal plasma has been estimated to be 6.9±0.6 mg/100 ml (≃ 1 μM). α_2PI is able to inhibit several different "serine" proteinases, but its main function is to inhibit plasmin-mediated fibrinolysis (for reviews see references 2–4). α_2PI efficiently inhibits the physiologically occurring fibrinolytic process that follows fibrin formation, thus stabilizing hemostatic plugs. The efficient inhibition of fibrinolysis is caused by the combined effects of three functional properties peculiar to α_2PI. These are the instantaneous inhibition of free plasmin, its interference with binding of plasminogen or plasmin to fibrin, and its susceptibility to Factor XIII-catalyzed cross-linking to fibrin (reviewed in references 4 and 5). Its physiological importance was established by the discovery of cases of congenital deficiency of α_2PI (6, 7) in which hemostatic plugs are dissolved prematurely before the restoration of injured vessels, resulting in a severe hemorrhagic tendency. So far, several pedigrees with congenital deficiency (reviewed in references 8 and 9) and one pedigree with molecular abnormality (10) of α_2PI have been reported.

Studies from our laboratories (11) and those of others (12, 13) have led to the isolation of the cDNA coding for human α_2PI. Recently, using the cDNA as a probe, Holmes et al. (14) have cloned a portion of the α_2PI gene from patients with abnormal α_2PI, α_2-antiplasmin Enschede, and identified an alanine insertion near the reactive site region of the molecule as its molecular defect. However, nothing was known about the molecular basis for the patients with congenital α_2PI deficiency with no detectable circulating antigen. Since we have isolated and characterized the human α_2PI gene (15), it has now become possible to analyze the gene from these patients with α_2PI deficiency. Therefore, this study was undertaken to elucidate the molecular basis for a pedigree of hereditary α_2PI deficiency by analyzing the α_2PI gene from an individual homozygous for this trait (16). This led to the identification of a single cytidine nucleotide insertion at nucleotide number 1438 in exon X, which should be the molecular basis for α_2PI deficiency in this pedigree.

Methods

Subjects. We studied a Japanese family with congenital α_2PI deficiency that was previously reported by Yoshioka et al. (16) (Fig. 1). The three sisters homozygous for the defect had functionally and immunologically no detectable α_2PI in their plasma, and the other heterozygous members including both parents had ~ 50% the normal concentration of α_2PI. Family history did not reveal any consanguinity (16).

Genomic Southern blot analysis. Genomic DNA was prepared from the peripheral leukocytes obtained from the six members with the pedigree and from normal controls, according to an established method (17). 5 μg of DNA samples were digested with various restriction endonucleases (Bam HI, Eco RI, Hind III, Pst I, Pvu II, Sac I, and Sma I). Agarose gel electrophoresis, transfer to nitrocellulose filters, hybridization, washing of filters and autoradiography were carried out by standard procedures (18). For use as probes, a 750 base pair (bp) Eco RI/Hind III fragment of the human α_2PI cDNA clone pPI39 (11), coding for the carboxyl-terminal half of α_2PI, and an 890 bp Pst I fragment of the human genomic α_2PI clone λPI6 (15), containing exons II, III, and IV, were labeled with α-[^{32}P]dCTP by nick translation.

Gene cloning and sequence analysis. Genomic DNA was isolated from an Epstein-Barr virus transformed lymphoblastoid cell line (19) derived from a homozygote (II-2 in Fig. 1) of the pedigree. About 100 μg of DNA was digested to completion with the restriction endonuclease Bam HI. The restriction fragments were separated according to size on a 0.8% agarose gel next to molecular weight markers. The gel was stained with ethidium bromide, and the DNA fragments in the 11–13 kilobase (kb) range were recovered from the gel by transfer to DEAE-

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1. *Abbreviations used in this paper:* α_2PI, α_2-plasmin inhibitor.

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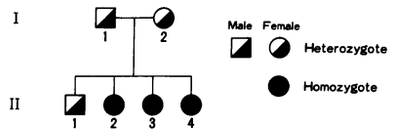

Figure 1. Pedigree of the family with congenital α_2PI deficiency.

cellulose paper (DE81 paper; Whatman Inc., Clifton, NJ) as described (20), since all the coding exons of the α_2PI gene were known to be contained in a 12-kb Bam HI fragment (15). About 0.3 μg of the size fractionated genomic DNA fragments were ligated to 1 μg of Bam HI digested EMBL3 phage arms (Strategene Cloning Systems, San Diego, CA) (21). The ligation reaction was performed overnight at 15°C in 5 μl of ligase buffer (50 mM Tris, pH 7.5; 10 mM $MgCl_2$; 10 mM dithiothreitol; and 1 mM ATP) with 1 Weiss unit of T4 DNA ligase. 4 μl of the ligation mixture was packaged in bacteriophage particles using a combination of freeze-thaw and sonic phage lysates (Gigapack; Strategene Cloning Systems) according to the manufacturer's instructions. The λEMBL3 library was grown in *Escherichia coli* strain P2392 (Strategene Cloning Systems). Phage plaques were screened by in situ hybridization with the nick translated cDNA probe according to the standard technique (18). Positive clones were plaque purified, and DNA isolated from confluent plate lysates was digested with Bam HI and ligated into the pUC18 plasmid that had been linearized by Bam HI digestion. Restriction fragments suitable for sequencing all the coding exons and exon-intron junctions were subcloned into the appropriate restriction sites of pUC18 to be sequenced by the dideoxy method using pUC vectors as described by Hattori and Sakaki (22). A region with multiple cytidine nucleotides in a row was also sequenced with the dideoxy method using avian myeloblastosis virus reverse transcriptase instead of Klenow DNA polymerase, as previously described (23).

Oligonucleotide probe hybridization analysis. Oligonucleotide probes were synthesized essentially as described by Studencki and Wallace (24). Two 19-mer templates and an 8-mer primer, complementary to the identical 3′ ends of the templates, were chemically synthesized by the phosphite method. The primer was first phosphorylated, and two 19-mer oligonucleotide probes labelled with α-[^{32}P]-dCTP were then synthesized on the templates by extension of the hybridized primer with DNA polymerase I (Klenow fragment). The labeled probes were then separated from the unphosphorylated templates on a 20% polyacrylamide urea gel.

Genomic DNA samples (10 μg) were digested to completion with both Eco RI and Bam HI, electrophoresed on 1% agarose gels, denatured, neutralized, and transferred to nylon membranes by standard procedures (18). The membranes were prehybridized for 1 h at 50°C in 6× standard saline citrate (SSC: 1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (Denhardt's solution contains 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 5% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 50°C in the same solution containing a ^{32}P-labeled oligonucleotide probe (5×10^5 cpm/ml). The membranes were washed three times for 30 min at room temperature in 6× SSC and 1% SDS and once for 30 min at 58°C in the same solution. Autoradiography was done with intensification screens at −70°C for 7 d.

Construction of expression vectors. An expression vector for the normal α_2PI, pSV2PI (Fig. 2), was constructed by inserting a genomic fragment and a cDNA fragment coding for the amino- and carboxyl-terminal halves of α_2PI, respectively, into the pSV2 vector, as follows: The Alu I-Eco RI fragment, containing the exons II to VI and the 5′ portion of exon VII, was obtained by Eco RI and partial Alu I digestion of the genomic clone λPI6 and inserted between the Sma I and the Eco RI sites within the multiple cloning region of pUC18. After the 20-bp region between the Alu I site and exon II was confirmed by sequencing, the genomic fragment was excised from the pUC18 vector along with the multiple cloning site sequence by Hind III-Eco RI digestion and then ligated into the Hind III and Eco RI sites of the pSV2 vector (25),

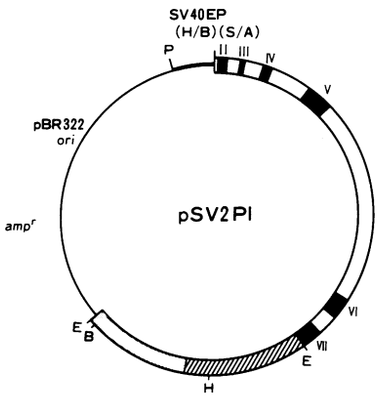

Figure 2. Structure of the α_2PI expression vector pSV2PI. The exons (*solid black regions*) in the genomic segment code the NH_2-terminal half of α_2PI, and the cDNA covers the rest of the coding sequence (*hatched segment*), stop signal, 3′ noncoding region (*dotted segment*) and polyadenylation signal. The exon numbers are indicated by the roman numerals. Open segment represents the introns of the α_2PI gene. The thin and thick lines reveal parts of the pSV2 vector derived from pBR322 and the SV40 DNA, respectively. (H)/(B) and (S)/(A) indicate fusions of the blunt-ended restriction sites shown in the parentheses. Abbreviations: pBR322 *ori*, the origin of replication of pBR322; *amp^r*, ampicillin resistance gene; SV40EP, SV40 early promotor region; A, Alu I; B, Bam HI; E, Eco RI; H, Hind III; P, Pvu II; S, Sma I.

which contained the replication origin, the enhancer and promoter sequences of SV40 DNA. To delete the multiple cloning site sequence and to eliminate the Hind III and Bam HI sites from this plasmid, it was digested with Hind III and Bam HI, filled-in with Klenow enzyme and religated with its blunt ends. Then, the 1.7-kb Eco RI fragment containing the rest of the coding region, stop signal, 3′ noncoding region and polyadenylation signal was excised from the α_2PI cDNA clone, pPI39 (11), and ligated into the Eco RI site of this plasmid. The insert direction was confirmed by digestion with several restriction enzymes.

To test the effect of the frameshift mutation identified in this pedigree, the 700-bp Hind III-Bam HI fragment of the abnormal α_2PI genomic clone, λαPN4 (Fig. 3), containing this mutation was exchanged with the corresponding fragment of pSV2PI to yield pSV2PN. The mutated sequence of pSV2PN was confirmed by the oligonucleotide probe described above.

Expression in heterologous cells. Monkey COS-7 cells (26) were maintained in DME containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

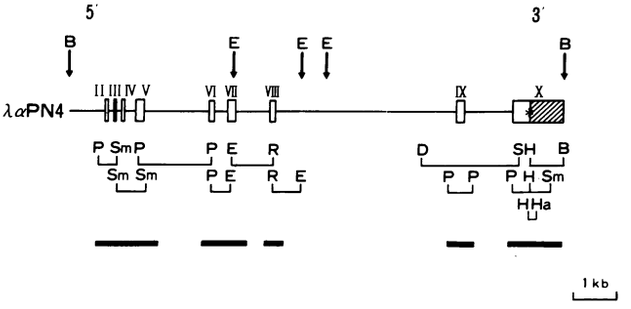

Figure 3. Structure and sequencing strategy of the α_2PI gene isolated from a homozygote (II-2) of the pedigree. Exons are represented by the open boxes and introns by the horizontal line. The exon numbers are indicated by the roman numerals. The position of the frameshift mutation in exon X is marked with an asterisk. The restriction enzyme fragments indicated below the gene were subcloned into pUC18 for sequencing. The lowermost bold lines indicate regions sequenced. The restriction sites indicated are: B, Bam HI; D, Dra I; E, Eco RI; H, Hind III; P, Pst I; R, Rsa I; S, Sac I; Sm, Sma I.

For transient expression assays, the plasmid DNAs were introduced into COS-7 cells by the DEAE-dextran-mediated transfection procedure as described (27). In brief, 5 μg of pSV2PI or pSV2PN were added per 100-mm dish of COS-7 cells which had been plated on the previous day (3×10^6 cells/plate). At 72 h after transfection, cytoplasmic RNA was harvested by a standard method (18), culture media were collected and centrifuged to remove cell debris, and the cells were harvested with trypsin, washed with PBS, resuspended in PBS, and lysed by two cycles of freeze-thawing. To measure the transcript levels of the introduced expression vectors, cytoplasmic RNA samples were quantitated by absorbance at 260 nm, subjected to formaldehyde-agarose gel electrophoresis and transferred to a nylon filter for RNA blot analysis using the α_2PI cDNA probe, essentially as described (18). α_2PI in the culture media and in the cell extracts was measured by an ELISA (28), using JTPI-1, a murine monoclonal antibody directed against the reactive site of α_2PI, as a solid-phase antibody, and JTPI-2 or JTPI-3, a monoclonal antibody recognizing an epitope between the carboxyl-terminal 26-residue peptide and the reactive site or an epitope near the amino-terminus, respectively, as a liquid-phase antibody.

Immunoprecipitation of [^{35}S]methionine-labeled recombinant α_2PI. COS-7 cells plated on the previous day (1×10^6 cells/60 mm plate) were transfected with pSV2PI, pSV2PN or, as a negative control, pSV2*neo* (25) as described above. At 48 h after transfection, the cells were washed with PBS and incubated with 1 ml of methionine-free DME with 2% dialyzed FCS. After 1 h of incubation at 37°C, the cells were labeled with 100 μCi of [^{35}S]methionine in 1 ml of methionine-free DME with 2% dialyzed FCS. After the labeling period of 15 min, cell extracts were prepared for immunoprecipitation of the intracellular recombinant α_2PI, or the cells were further incubated for 2 h in DME with 2% FCS supplemented with 250 μg/ml methionine for immunoprecipitation of the recombinant α_2PI in the culture medium. Before immunoprecipitation, the cells were washed with PBS and solubilized in 2 ml of ice-cold lysis buffer (140 mM NaCl; 10 mM Tris, pH 7.4; 1 mM EDTA; 0.5% sodium deoxycholate; 1% NP40; and 1 mM phenylmethanesulfonyl fluoride), and the solubilized material was clarified by centrifugation at 10,000 *g* for 1 h at 4°C. The conditioned medium was clarified by centrifugation at 10,000 *g* for 10 min at 4°C before immunoprecipitation.

For immunoprecipitation, 300 μl of lysate or conditioned medium was incubated with 10 μl of 1 mg/ml goat polyclonal anti-human α_2PI immunoglobulin (Biopool, Umea, Sweden). After 2 h of incubation at 4°C, 20 μl of 50% recombinant protein G-agarose (Zymed Laboratories, San Francisco, CA) was added to samples and incubated for 1 h at 4°C with intermittent shaking. Immunoprecipitates were washed four times with the lysis buffer supplemented with 0.1% SDS to remove nonspecifically adsorbed proteins. Bound antigen was eluted from the beads by heating at 95°C for 5 min in SDS sample buffer (63 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 150 mM dithiothreitol; and 0.04% bromophenol blue) and then subjected to electrophoresis on 10% SDS-polyacrylamide gels (29), followed by treatment with enhancer (Enlightning; New England Nuclear, Boston, MA), drying, and fluorography. The molecular weight standards used were ^{14}C-methylated myosin (200 kD), phosphorylase-b (92.5 kD), BSA (69 kD), and ovalbumin (46 kD).

Results

Southern blot analysis. The human α_2PI gene has a deletion-insertion type restriction fragment length polymorphism (RFLP) in intron 8 (unpublished observation). Purified DNA samples from the family members were doubly digested with Eco RI/Bam HI and analyzed with the cDNA probe for this RFLP. Fig. 4 shows that the heterozygotes of the pedigree had both of the variable (polymorphic) fragments, 5.3 and 4.6 kb, but the homozygotes had only the 5.3-kb one, indicating that the defective α_2PI gene of this pedigree is associated with the

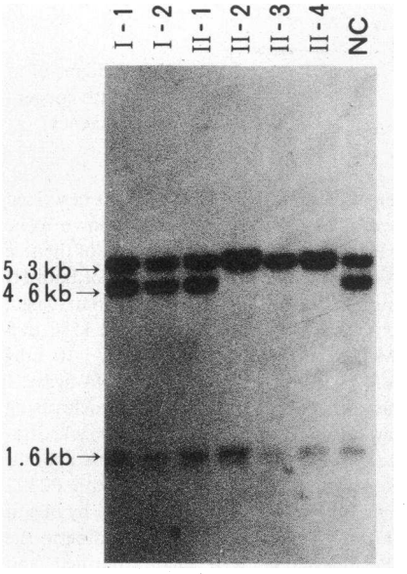

Figure 4. Demonstration of the RFLP in the α_2PI genes of the family members. DNA from the family members (I-1 to II-4 in Fig. 1) and from a normal control (NC) was digested with both Eco RI and Bam HI, and analyzed by the Southern Blot analysis using the α_2PI cDNA probe. The numbers at the left indicate the sizes of the fragments in kilobases (kb).

longer fragment. When the DNA sample from a homozygous individual of this pedigree was digested with one of the seven various restriction enzymes (listed in Methods) and compared with that of a normal individual who was also homozygous for the larger fragment of the RFLP, no deletion or rearrangement was detected by Southern blot analysis using either the cDNA or genomic DNA probes (data not shown).

Gene cloning and sequence analysis. A λ-phage library was constructed from the genomic DNA from a homozygote (II-2) of the pedigree, as described in Methods. About 2.0×10^5 plaques of the λEMBL3 library were screened, and one out of four positive clones obtained was analyzed. The isolated recombinant λEMBL3 clone, λαPN4, containing the α_2PI gene sequence of the homozygote (II-2) was mapped (Fig. 3) and compared with the normal clones previously characterized (15). Except for the difference in length of intron 8, which accounted for the RFLP described above, we did not find any other differences between the maps, which further confirmed the results of Southern blot analysis that denied gross deletion and rearrangement.

After separately subcloning 9 exon regions as shown in Fig. 3, we sequenced all the exons and the exon-intron boundaries except for the first noncoding exon (15), which was not included in the clone λαPN4. The sequence was found to be identical with the normal α_2PI gene with only one exception: near the 3′ end of the coding region in the last exon, in the region coding for the plasmin(ogen) binding site, a cytidine nucleotide was found to be inserted at nucleotide position 1438, at a position just downstream of six consecutive cytidine nucleotides (Fig. 5). This sequence change was confirmed by sequencing both strands of the DNA. This insertion would lead to substitution and extension of the carboxyl-terminal 12 normal amino acid residues by causing shift in the reading frame of the messenger RNA and allowing it to be read through the normal termination codon until the next chain termination signal is reached, as shown in Fig. 6.

Oligonucleotide probe hybridization analysis. To directly analyze DNA samples from the family members, we synthesized two oligonucleotide probes designed to detect this mutation (see Fig. 7, legend). As shown in Fig. 7, the 5.3-kb Eco

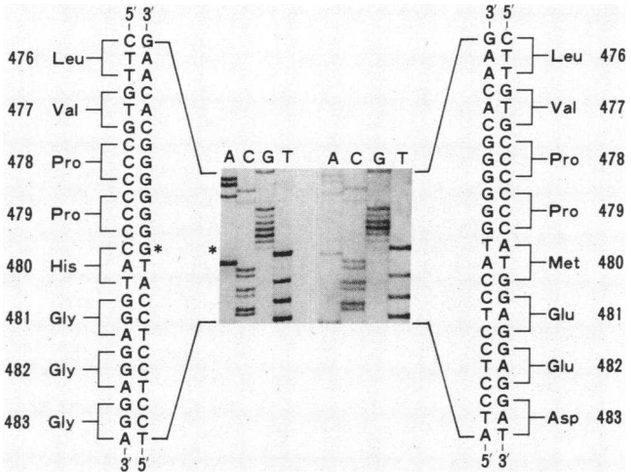

Figure 5. Difference in the sequence of the α_2PI gene isolated from a homozygote (II-2) and the normal gene. (*Left*) Nucleotide sequence of the non-coding strand near the 3′ end of the coding region in exon X of the α_2PI gene isolated from the homozygote. (*Right*) The sequence of the same region of the normal gene. The nucleotide sequences are indicated along the corresponding amino acids and residue numbers. Nucleotide C (G in the noncoding strand indicated with asterisks) is inserted in the coding strand at a position just downstream of six consecutive C nucleotides, causing a frameshift. This sequence with multiple C nucleotides in a row was determined by the dideoxy-method using avian myeloblastosis virus reverse transcriptase, as described in Methods.

RI/Bam HI fragments of all the family members affected hybridized only with the mutant probe, while the 5.3-kb or the 4.6-kb fragment of a normal control and the 4.6-kb fragment of the heterozygotes hybridized only with the normal probe. Thus, in addition to confirming that the frameshift mutation characterized was not a cloning artifact, these probes revealed that the other members affected, including both parents, had the same mutation in their α_2PI genes associated with the 5.3-kb Eco RI/Bam HI fragment and that the homozygotes were also homozygous for this mutation. These results suggest that this mutation is the cause of α_2PI deficiency in this pedigree.

Expression in heterologous cells. To ensure that the frameshift mutation identified in this pedigree could cause the deficiency of α_2PI and to elucidate its mechanism, we constructed an α_2PI expression vector, pSV2PN, containing the abnormal sequence, as described in Methods, and transfected it into COS-7 cells for a transient expression assay. RNA blot analysis of the cytoplasmic RNA of COS-7 cells did not reveal any significant difference between the transcript levels of a normal α_2PI expression vector, pSV2PI, and a mutant α_2PI expression vector, pSV2PN (Fig. 8). An analysis for α_2PI in the culture media and in the cell extracts, assayed by ELISA using monoclonal antibodies JTPI-1 and JTPI-2, is shown in Table I. These data indicate that ~ 99% of the normal recombinant α_2PI that was synthesized in the COS-7 cells was secreted into the culture medium and that the level of the mutant α_2PI secreted into the medium was ~ 4% of the level of the normal

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                                480                                                500
 G  P  D  L  K  L  V  P  P  H  G  G  G  L  P  P  V  W  Q  P  Q  V  R  G  R  G  C  G  I  Q
GGCCCTGACTTAAAACTTGTGCCCCCCCATGGAGGAGGATTACCCCCAGTTTGGCAGCCCCAAGTGAGGGGCCGTGGCTGTGGCATCCAG
 G  P  D  L  K  L  V  P  P  *  M  E  E  D  Y  P  Q  F  G  S  P  K
                          (1440)                                                   (1500)

                                                520
 S  P  C  L  D  Q  P  L  H  S  C  D  S  F  Q  P  A  L  W  H  W  G  R  G  R  G  Q  S  E  R
AGTCCCTGCCTGGACCAGCCTCTCCACTCATGTGACTCTTTCCAACCGGCTTTGTGGCACTGGGGCAGGGGCCGGGGGCAGTCTGAGAGA
                                                (1560)

                                540                                                560
 G  H  S  F  P  T  P  L  G  E  F  R  V  G  G  G  R  G  W  E  E  G  R  H  R  G  A  G  S  L
GGCCATTCTTTCCCAACACCTCTTGGGGAGTTTAGGGTGGGGGGGGGGCGCGGCTGGGAGGAGGGCAGGCATCGGGGAGCCGGGAGCCTG
                          (1620)                                                   (1680)

                                                580
 T  L  I  F  L  P  N  R  L  R  G  C  P  A  P  G  P  G  Q  E  G  G  A  S  S  S  A  R  R  Q
ACCCTCATCTTTCTTCCAAACAGGCTCAGAGGGTGTCCTGCACCGGGGCCTGGGCAGGAGGGAGGTGCTTCTAGTTCTGCCAGGAGACAG
                                                (1740)

                                600                                                620
 V  S  C  S  P  R  Q  L  G  H  P  D  F  C  L  P  E  K  K  G  G  G  E  G  C  L  W  T  C  P
GTTAGCTGCTCCCCACGTCAGCTGGGACACCCCGACTTTTGTTTACCAGAGAAAAAGGGAGGGGGAGAGGGCTGCCTTTGGACTTGTCCC
                          (1800)                                                   (1860)

                                                640
 G  T  P  R  L  G  W  G  E  T  G  P  G  G  G  S  G  G  E  A  L  S  S  A  P  R  G  T  R  V
GGGACACCTAGGCTAGGGTGGGGAGAGACGGGCCCTGGTGGTGGCTCGGGAGGCGAAGCGTTGTCCTCAGCCCCGCGTGGAACTCGTGTC
                                                (1920)

 W  H  S  L  A  V  A
TGGCACAGCCTGGCTGTGGCCTAACCTGCCGAGAGTCCATCAGCCTCCATCCTACCCCCTGTGCCTTGTCACGCCAGACTTCCCACGGCT
                          (1980)
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Figure 6. Effect of the single nucleotide insertion on the deduced amino acid sequence of the carboxyl-terminal portion of the α_2PI. A portion of the nucleotide sequence of the mutated α_2PI gene that has been cloned is shown with nucleotides numbered in parentheses. The numbering of the nucleotides was started at the initiation codon and only the bases in the exons have been numbered. The deduced amino acid sequence of the mutated gene is displayed above the nucleotide sequence with amino acid numbers, which have also started with the initiator methionine. The amino acid sequence of the normal α_2PI is shown beneath the nucleotide sequence. The termination codons are underlined. As a result of the cytidine nucleotide insertion indicated by an asterisk, a frameshift occurs that leads to replacement of the carboxyl-terminal 12 amino acid residues of the normal α_2PI with 178 amino acid residues entirely unrelated to the normal amino acid sequence.

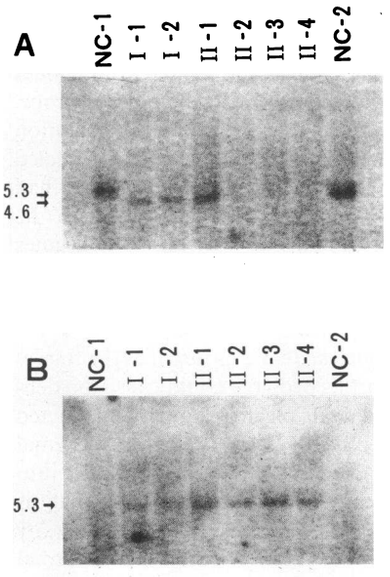

Figure 7. Detection of the frameshift mutation in the α_2PI gene by oligonucleotide hybridization studies. Genomic DNA from the family members (I-1 to II-4) and that from normal controls (NC-1,2) was digested with both Eco RI and Bam HI, electrophoresed through agarose, transferred to nylon membranes and hybridized with oligonucleotide probes synthesized by the primer extension reaction, as described in Methods. Normal controls, NC-1 and NC-2, were homozygous for the 5.3- and 4.6-kb Eco RI/Bam HI fragments, respectively. In A, the normal 19-mer probe (5′ ACTTGTGCCCCCCATGGAG 3′) hybridized to the Eco RI/Bam HI restriction fragments in digests of DNA isolated from normal individuals (NC-1,2) and only the 4.6-kb fragments of the heterozygotes of the pedigree (I-1,2, II-1). In B, the 19-mer mutant probe (5′ ACTTGTGCCCCCCCATGGA 3′), corresponding to the frameshift mutation shown in Fig. 5, hybridized only to the 5.3 kb Eco RI/Bam HI fragments in digests of DNA isolated from both heterozygotes (I-1,2, II-1) and homozygotes (II-2,3,4,) of the pedigree.

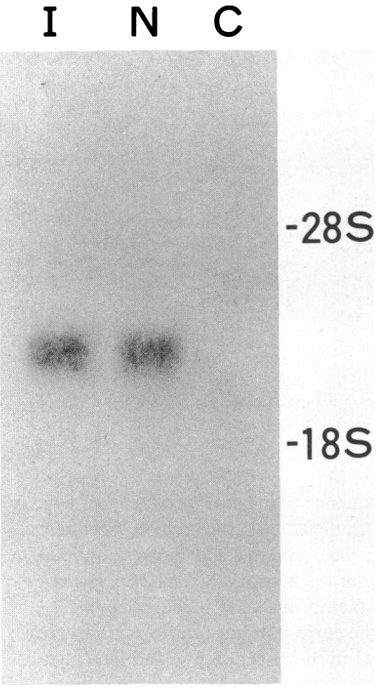

Figure 8. RNA blot analysis of the transcript levels of pSV2PI and pSV2PN transfected into COS-7 cells. At 72 h after transfection, cytoplasmic RNA was isolated, and 10 μg each of the samples were subjected to formaldehyde-agarose gel electrophoresis and transferred to a nylon filter for RNA blot analysis using the α_2PI cDNA probe. Lanes *I* and *N* correspond to RNA samples isolated from COS-7 cells transfected with pSV2PI and pSV2PN, respectively. Lane *C* represents RNA isolated from untransfected COS-7 cells. Similar amounts of ethidium bromide-stained 28S and 18S RNA were visualized in each lane. The positions of ribosomal RNA size markers are indicated at the right margin.

recombinant protein secreted although their cellular contents did not differ significantly. To exclude any possible effect of the altered carboxyl-terminal portion of mutated α_2PI on the ELISA using JTPI-2, which recognizes an epitope near the carboxyl-terminus, we also measured α_2PI in the culture media by ELISA using JTPI-3, which recognizes an epitope near the amino-terminus; the obtained results were consistent with those shown in Table I (data not shown).

To confirm that the elongated α_2PI as predicted from the nucleotide sequence data is really synthesized in the COS-7 cells transfected with pSV2PN, COS-7 cells transfected with pSV2PI, pSV2PN, or pSV2*neo* as a control were biosynthetically labeled with [^{35}S]methionine. The α_2PI immunoprecipitated from these cells or culture media was subjected to SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 9). When the intracellular α_2PIs, expected to be the precursor forms, are compared, the difference in size ($\simeq$ 16 kD) between the normal and mutant proteins was in agreement with the value (17 kD) predicted from the deduced amino acid sequences shown in Fig. 6.

Discussion

To elucidate the molecular genetic basis for the congenital deficiency of α_2PI, we have cloned and sequenced the α_2PI gene of a homozygote. The sequence was compared with the normal sequence, and a frameshift mutation near the 3′ end of the coding region was found as the sole significant sequence change. The elongation of amino acid sequence caused by the frameshift mutation in the mutant α_2PI (Fig. 6) was confirmed by an analysis of biosynthetically labeled α_2PI (Fig. 9). Frameshift mutations have been also reported in β-thalassemias (reviewed in 30, 31), hemophilia B (32), and α_1-antitrypsin deficiency (33, 34) in which no gene products have been detected. However, how these frameshift mutations cause the deficiencies has not been well defined. Because it is not feasible to obtain the hepatocytes, synthesizing α_2PI in normal individuals, from our patients to study the in vivo effect of the mutation, we carried out experiments in which the mutated gene was transfected into cultured cells for expression to study the mechanism(s) by which this frameshift mutation leads to the deficiency. Our transfection experiment data provide strong evidence for the impairment of the secretory process of the mutant α_2PI, for this mutation significantly reduced the amount of the mutant α_2PI secreted into the medium, while the cellular content was comparable to that of normal recom-

Table I. Expression and Secretion of Normal and Mutated Recombinant α_2-Plasmin Inhibitor from COS Cells

Plasmid	α_2PI in media	α_2PI in Cell
	ng	*ng*
None	0	0
pSV2PI	1,864	27
pSV2PN	83	28

Culture media and cells were harvested from 10-cm plates 72 h after transfection with α_2PI expression plasmids. α_2PI in the culture media and in the cell extracts was measured by ELISA using JTPI-1 as a solid-phase antibody and JTPI-2 as a liquid-phase antibody as described in Methods.

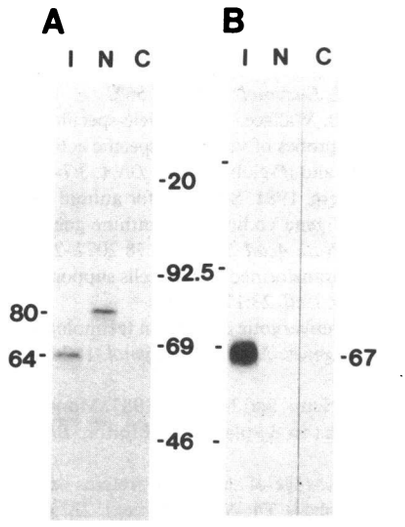

Figure 9. SDS polyacrylamide gel electrophoresis of ^{35}S methionine-labeled recombinant α_2PI immunoprecipitated from the COS-7 cells and conditioned medium. COS-7 cells were transfected with pSV2PI (*I*), pSV2PN (*N*), or pSV2*neo* (*C*). At 48 h after transfection, the cells were labeled with 100 μCi/ml [^{35}S]methionine for 15 min. Cell lysates (*A*) or conditioned media (*B*) harvested after 2 h incubation of the labeled cells in methionine-rich medium were immunoprecipitated with goat anti-human α_2PI immunoglobulin and subjected to electrophoresis under reducing conditions on 10% SDS-polyacrylamide gels. The positions of the normal recombinant α_2PI in the cells (64 kD) or in the conditioned medium (67 kD) and the position of the mutant α_2PI in the cells (80 kD) are indicated. The band corresponding to the mutant α_2PI in the conditioned medium (*B*, column *N*) is faintly visible at the position coinciding with that of the mutant α_2PI in the cells (80 kD). Molecular weight markers are indicated at the center.

binant α_2PI (shown in Table I). The impairment of the secretion is not unexpected, since there is much evidence that abnormal secretory or plasma membrane proteins often are specifically retained within the rough endoplasmic reticulum and are not secreted, unless they fold into native or near-native conformations within the rough endoplasmic reticulum (recently reviewed in reference 35). In the present case, the frameshift mutation leads to the alteration and elongation of the α_2PI (Figs. 6 and 9), which would cause a substantial change in its secondary and tertiary structure; thus, its posttranslational transport through intracellular secretory compartments should be affected. The in vitro study also suggest the instability of the mutant α_2PI, possibly also due to the altered conformation, because no abnormal intracellular accumulation of the α_2PI was observed in spite of the apparently retarded secretion. This mutation did not have any significant effect on the transcript level as tested with the RNA blot analysis in the heterologous transfection system, although similar elongated translation caused by a termination codon mutation has been reported in thalassemic hemoglobinopathy in which deficient globin chain synthesis was caused by the decrease in its unstable mRNA (36). When the plasma α_2PI levels of the homozygotes were measured by ELISA using JTPI-1 and JTPI-2, they were much lower (0.3–0.5 μg/ml or $\sim$ 0.4–0.7% of the normal level, our unpublished data) than that expected from the transfection data ($\sim$ 4% of the normal level). Several possible explanations for this discrepancy could be offered; the expression and/or secretion in cultured COS-7 cells may be quite different from those occurring in the liver cells in vivo, or the mutant α_2PI may be more rapidly removed from the circulation in vivo because of its unstability in plasma or its higher susceptibility to in vivo clearance mechanism.

In the present case, a cytidine nucleotide was inserted in the DNA sequence of six consecutive cytidine nucleotides. This is in accordance with the DNA slippage hypothesis of frameshift mutations (37), for this theory predicts that frameshift mutations occur particularly at runs of identical bases in the DNA, where displacement ("looping out") of bases from either the template strand (giving a deletion) or the growing strand (giving an insertion) can be stabilized by normal base paring beyond the unpaired base in the replication process (38).

Synthetic oligonucleotide probes revealed the presence of this mutation in the α_2PI gene of both heterozygous parents, although they shared no relatives for at least four generations (16). One possible explanation for this finding is that there are two separate independent origins of the identical mutation. In this regard, it is worthy to note that this mutation site may be prone to frameshift mutations as discussed. Another more likely explanation is the occurrence of this mutation in a possible common ancestor of the two parents, since their ancestors on both sides came from the same island called Amami-Ohshima located in the southwestern part of Japan. Then, without any apparent disadvantage of heterozygous deficiency, the mutated allele might be distributed over and even outside the island, although at a low frequency. This supposition requires further testing with these oligonucleotide probes, particularly of other deficiency patients.

Since we have not sequenced the whole α_2PI gene from the patient, it cannot be ruled out that other mutations not detected by the Southern blot analysis might be present elsewhere in the gene. However, we infer that the primary and likely the only cause of α_2PI deficiency in this pedigree is this frameshift mutation for two reasons. First, complete deficiency caused by point mutations in DNA sequences other than amino acid coding regions or exon-intron boundaries has been seldom, if ever (39), reported even in cases of thalassemias, which have been molecular biologically studied in detail. Second, it is not necessary to hypothesize any additional mutations to explain the deficiency, since the frameshift mutation reported here seems to sufficiently account for it.

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