Structural Organization of the Gene for Human Prolidase (Peptidase D) and Demonstration of a Partial Gene Deletion in a Patient with Prolidase Deficiency*

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Prolidase (peptidase D, iminodipeptidase, EC 3.4.13.9) is an ubiquitous enzyme which splits dipeptides with a prolyl residue in the carboxyl-terminal position. The enzyme is expressed in all tissues and cells in humans. The induction or hormonal control of the enzyme activity under various conditions is not well understood. The enzyme has been isolated from various sources, including humans (1-3). The human enzyme is a homo-dimer with a subunit of 56,000 daltons (3, 4), and the activity is activated by a manganese ion.

We isolated cDNA clones corresponding to human prolidase (5) and determined the primary structure of the subunit polypeptide (6). The subunit protein is composed of 492 amino acid residues and is rich in glutamic acid residues (6). The prolyl residue in the carboxyl-terminal position is acylated, and there are two putative glycosylation sites (6). We also determined chromosomal localization of prolidase (6).

Deficiency of the enzyme results in abnormalities of the skin and other connective tissues (7). The affected subjects excrete massive amounts of iminopeptides into the urine, and it is these peptides which function as a substrate for prolidase (8). This rare genetic prolidase deficiency is inherited as an autosomal recessive trait (9). In foregoing work, we defined the polypeptide and RNA phenotypes of cells obtained from patients with prolidase deficiency (10). All these analyses revealed that the genetic defects in these patients are heterogeneous.

To comprehend the structure-function relationships, gene organization, and biosynthetic regulatory mechanisms better, we isolated and characterized the gene for human prolidase. This gene is over 130 kilobases (kb) long and consists of 15 exons. The absence of the 14th exon was noted in a Japanese girl, who was a product of consanguineous mating and was deficient in prolidase.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan) and from Toyobo Co. (Osaka, Japan) and were used essentially as recommended by the suppliers. M13 sequencing kit, random primer DNA labeling kit, T4 polynucleotide kinase, and M. Mori, Kumamoto University) (14). Approximately 1 X 10^6 EMBL4 phages of each total DNA library were screened, using the human prolidase cDNA insert of PL21 as a probe (6). For chromosome walking, we screened the Sau3A partial library, using appropriate nuclear highly repetitive, sequence-free DNA fragments derived from some of the isolated clones. Phage DNAs of positive clones were radiolabeled with [α-32P]dCTP (3,000 Ci/mmol) by multiprimer priming (12).

Isolation and Characterization of Phage Clones Containing the Human Prolidase Gene—Two independently constructed human genomic DNA libraries were screened for clones carrying the prolidase gene using the plaque hybridization technique (13). The first was constructed from EcoRI partial digests of human liver DNA (a kind gift from Dr. A. Mita and Dr. K. Shinoda, Kumamoto University), and the second was constructed from Sau3A partial digests of human leukocytes DNA (a kind gift from Drs. M. Takiguchi, Y. Haraguchi, and M. Mori, Kumamoto University) (14). Approximately 1 X 10^6 EMBL4 phages of each total DNA library were screened, using the human prolidase cDNA insert of PL21 as a probe (6). For chromosome walking, we screened the Sau3A partial library, using appropriate nuclear highly repetitive, sequence-free DNA fragments derived from some of the isolated clones. Phage DNAs of positive clones were

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The abbreviations used are: kb, kilobase(s); RFLP, restriction fragment length polymorphism; PIPES, piperezine-N,N'-bis(3-ethanesulfonic acid); SDS, sodium dodecyl sulfate; bp, basepair.
characterized by restriction mapping and Southern hybridization analysis. The genomic DNA inserts in the purified phages were excised by digestion with EcoRI and BamHI for subsequent restriction mapping and subcloning.

**DNA Sequencing Analysis**—Subclones were constructed with plasmid pUC18 as a vector, and the nucleotide sequence of the double-stranded plasmid subclones were determined by the dideoxynucleotide chain termination method (15, 16), using synthetic oligonucleotide primers complementary to the sequence of the vector or the prolidase cDNA sequence. Oligonucleotide primers were synthesized using a DNA synthesizer (Model 381A, Applied Biosystems, Foster City, CA).

**Primer Extension Analysis**—Poly(A)* RNA was prepared from human placenta by guanidium thiocyanate extraction (17) followed by phenol-chloroform extraction and ethanol precipitation. Primer extension analysis was performed as described (18). A synthetic oligonucleotide complementary to the human prolidase gene, nucleotide +114 to +133 relative to translation initiation site was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The labeled primers were hybridized to RNA by incubating for 20 min at 90 °C. After precipitation of the annealed primer and template, the hybridized primers were extended by incubation with 30 units of avian myeloblastosis virus reverse transcriptase in reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3; 8 mM MgCl2; 30 mM KCl; 5 mM dithiothreitol) containing 0.56 mM each dideoxynucleotide triphosphate. The sample was precipitated by the addition of ethanol, denatured, and analyzed on a 6% polyacrylamide gel.

**Nuclease S1 Mapping**—S1 nuclease protection of the 5' end of the mRNA was performed as described (19). A 242-bp fragment covering the putative transcription initiation site was synthesized by the polymerase chain reaction technique (20). Polymerase chain reaction technique was performed with a template of a 5.6-kb BamHI genomic fragment containing exon 1 and two kinds of primers, using TaqI DNA polymerase and DNA thermal cycler (Perkin-Elmer Cetus Instruments). A 5' primer is 25 bases (~106 to ~85) with the P&I site at position 5, and a 3' primer is 20 bases (~114 to ~133), complementary to exon 1.

The fragment was labeled at 5' terminus with [γ-32P]ATP and T4 polynucelotide kinase. After digestion with PstI, the [P-32P]-labeled 236-bp fragment was isolated by electrophoresis on a NuSieve GTG agarose (Takara Shuzo Co.) and used as a probe (7.0 × 10^6 cpn/pmol of DNA). Approximately 0.1 pmol of the probe was denatured in 50 μl of S1 hybridization solution (80% formamide; 40 mM PIPES, pH 6.4; 1 mM EDTA, 400 mM NaCl) with 8 μg of poly(A)* RNA at 80 °C for 10 min, then at 45 °C for 20 h.

The sample was digested with 500 units of S1 nuclease by incubating for 90 min at 30 °C in 500 μl of S1 buffer (30 mM sodium acetate, pH 4.8, 100 mM NaCl, 1 mM ZnSO4) containing 10 μg of denatured calf thymus DNA. The protected fragments were precipitated by the addition of ethanol, denatured, and analyzed on a 6% polyacrylamide gel.

**Southern Blot Analysis**—Human genomic DNA was prepared from peripheral white blood cells of disease-free individuals, and lymphoblastoid cells obtained from subjects with prolidase deficiency (10) were cultured as described (21). Enzyme activities in the lymphoblastoid cells were not detected and immunologically cross-reacting materials were absent (10). DNA (5–10 μg) was digested with restriction enzymes under conditions recommended by the manufacturers, fractionated by gel electrophoresis on 8% agarose, and transferred to nitrocellulose filters (22). The filters were hybridized in medium containing 6 × standard saline citrate (SSC) (1 × SSC denotes 0.15 M NaCl and 0.015 M trisodium citrate), 5 × Denhardt’s solution, 50% deionized formamide, 0.25% SDS, 100 μg/ml salmon sperm DNA, and a 1–2 × 10^6 cpn/ml of [P-32P]-labeled probe. After hybridization for 16–20 h at 42 °C, the filters were washed in 2 × SSC, 0.1% SDS at room temperature, and 1 × SSC, 0.1% SDS at 65 °C. Autoradiography of the dried filter was performed at ~80 °C with intensifying screens (Du Pont Instrument, Wilmington, DE).

**RESULTS**

**Isolation and Characterization of the Prolidase Gene**—Two phage libraries constructed from human liver and human leukocytes were screened for the prolidase gene. Approximately 42 independent clones were isolated and analyzed by restriction enzyme digestion and partial sequencing (Fig. 1). These clones overlapped, except for one region, and spanned over 140 kb. Since a part of intron 9 was not cloned completely, the gene must be larger than indicated in Fig. 1. To define positions and boundaries of the exon blocks, the restriction fragments identified by Southern hybridization were subcloned and their sequences determined (Fig. 1, Table I). The gene was divided into 15 exons which ranged from 45 bases (exon 7) to 538 (exon 15), and the 14 introns ranged in size from 1.0 kb (intron 3) to 50 kb longer (intron 9). Since the exons total about 1.9 kb, 98% of the gene is occupied by introns. We attempted to clone the entire intron 9 by chromosome walking, using appropriate DNA fragments derived from some of the isolated clones. We were not able to elucidate the structure of intron 9, even when additional human libraries (23) were screened.

All of the splice donor and acceptor sites conform to the GT/AG rule (24) for nucleotides immediately flanking exon borders (Table I). We found six nucleotide substitutions between the cDNA sequence (6) and genomic sequence; G to A at nucleotide position 793, T to G at 1063, A to T at 1101, G to A at 1294, C to T at 1874, and G to C at 1887, four of which were present in the coding region, and two in the 3'-untranslated region. All of four nucleotide substitutions present in the coding region caused amino acid changes.

**Characterization of the 5' and 3' Ends of the Prolidase Gene**—The nucleotide sequence around the 5' and 3' ends of the prolidase gene is shown in Fig. 2. The 5' end of the mRNA was determined by nuclease S1 mapping and primer extension analysis (Fig. 3) and is numbered +1. The DNA fragment labeled at position 2 downstream from the first nucleotide of the initiation triplet was used as a probe for the S1 mapping. We noted a protected gene fragment of 133 bases for placental poly(A)* RNA. The 133-base-long fragment, starting from the primer labeled at the same position as the S1 probe, was also detected by primer extension analysis. From these results, the 5' end of the prolidase mRNA was assigned to a position 131 bases upstream from the first nucleotide of the initiation triplet. The assigned 5' end was the residue A, the generally preferred cap site (24, 25). The sequence CAAT (−67 to −64), resembling canonical "CAAT" box is situated at the usual location, that is around 70–80 bases upstream from the cap site (25) (Fig. 2A). A sequence resembling the canonical "TATA" box was not observed. Seven sets of potential binding sites for the cellular transcription factor Sp1, CCGCCC (26) an inverted form of GGCGCG were present at positions −52 to −47, −36 to −33, −21 to −16, −11 to −6, +39 to +44, +51 to +56, and +62 to +67 (Fig. 2A). The 3'-untranslated region of the prolidase gene contains 393 nucleotides (Fig. 2B). The site of the polyadenylation signal was inferred from the cDNA sequence (6). A typical poly(A)* addition signal sequence AAATAA is located 21 bases upstream from the poly(A)* tail.

**Detection of Restriction Fragment Length Polymorphism**

FIG. 1. Physical map of the human prolidase gene. The structure of the gene is shown at the bar at the top of the diagram. Exons 1–15 are shown as vertical lines and are numbered. Below the gene structure, EcoRI and BamHI sites are shown. The genomic DNA fragments contained in the phage clones are shown below the restriction map.
TABLE I

Exon-intron organization of the gene for human prolidase

| Exon | Size (bp) | Intron Size | Exon Size (kb) | Exon Size (kb) |
|------|-----------|-------------|----------------|----------------|
| 1    | 148       | 1           | 9.5            | ACC GG Thr Gly |
| 2    | 184       | 2           | 1.6            | GGG GAG Arg Gin |
| 3    | 128       | 3           | 1.0            | GGA AA Gly Lys |
| 4    | 64        | 4           | 7.0            | GAT GAG Asp Glu |
| 5    | 48        | 5           | 4.5            | ACT TCG Thr Leu |
| 6    | 62        | 6           | 14.0           | AGC AA Ser Lys |
| 7    | 45        | 7           | 13.0           | GAG TG Glu Cys |
| 8    | 76        | 8           | 1.1            | GTG GAG Arg Glu |
| 9    | 47        | 9           | >50.0          | GAA AG Glu Ser |
| 10   | 69        | 10          | 3.0            | GCC AG Gly Ser |
| 11   | 78        | 11          | 8.8            | ATG TC Met Cys |
| 12   | 140       | 12          | 10.5           | CCA G Pro Gly |
| 13   | 185       | 13          | 2.7            | CCA GAG Pro Glu |
| 14   | 192       | 14          | 1.3            | GCC GGG Gly Lys |
| 15   | 528       |             |                | AAG TAG Lys Stop |

**NOTE:**
- Exon sequences are shown in capital letters, and intron sequences are in small letters.
- The numbers shown at the exon-intron junctions indicate the positions of the corresponding amino acids in the prolidase gene, as deduced from the prolidase cDNA sequence (6).

**TABLE II**

**RFLP in the Prolidase Gene**—To search for RFLPs in the prolidase gene, we analyzed DNAs of 15 nonconsanguineous, healthy individuals, by agarose gel electrophoresis and Southern blot analysis after digestion with more than 20 restriction enzymes, using as probes the cDNA insert of PL21 or various genomic DNA fragments. We noted RFLPs in KpnI-digested human genomic DNA and BamHI when the labeled cDNA was used as a probe. A 4.0-kb BamHI-EcoRI fragment (pG14) containing exons 14 and 15 had a polymorphism with EcoRV. These results are summarized in Table II.

**Southern Blot Analysis of Prolidase Deficiency**—We first analyzed DNA from three patients with prolidase deficiency by Southern blot analysis after TaqI or BamHI digestion, using the cDNA insert of PL21 as a probe. A re-arrangement such as a major deletion or insertion was never evident (data not shown). We then reexamined all exons, except for exon 6, using specific genomic DNA fragments containing each exon. We were not able to elucidate exon 6 by Southern blots when a 1.0-kb BamHI fragment containing exon 6 was used as a probe. This fragment we used may had repetitive sequences in the intron sequence. There were no re-arrangements in exons 1 to 13, except for exon 6 (data not shown).

As shown in Fig. 4, a 4.0-kb BamHI-EcoRI fragment of genomic DNA (pG14), which contained exons 14 and 15, hybridized with 4.4- and 1.5-kb fragments generated by TaqI digestion in the control (lane 1). On the other hand, a 1.3-kb fragment (instead of the 1.5-kb fragment) appeared when the DNA sample from a patient (lane 2) was analyzed. When the same samples were digested with BamHI and subjected to Southern hybridization with the same probe, a ~8.7-kb fragment was visualized in lanes where the DNA sample from the control and the patient were analyzed (lanes 3 and 4, respectively). PstI digested DNA from the patient had a normal 1.4-kb and a 1.1-kb fragment but lacked the normal 0.8-kb fragment (lane 6). Control experiments suggested these changes were not due to RFLPs. The patient was a product of consan-
Structure of the Human Peptidase D Gene

Fig. 2. Nucleotide sequence of 5' (A) and 3' (B) termini.
The sequence on both strands was determined. A, the underline (+1 to +148) denotes the first exon. The initiation codon is in squares. The boxed area with the symbols resemble the following sequences: "CAAT," CAAT box (25); Spl, Spl-binding site (26). B, the sequence downstream of the termination codon. TAG (squares) is shown. The putative poly(A) addition signal is boxed. The boxed urea with the symbols resemble the following sequences

guineous mating and her sister had a similar enzyme defect, as described elsewhere (10). Thus, the patient seemed to be homozygous. These findings revealed a partial gene deletion of several hundred bases, thereby eliminating exon 14, in this particular patient. A major abnormality in gene structure was not evident in the other patients studied.

DISCUSSION

We determined the structural organization of the human chromosomal gene for prolidase by analyzing the overlapping genomic clones obtained from two different human gene libraries. This 130-kb-long gene is one of the relatively large ones thus far determined (the dystrophine gene, 2000 kb (27); the cystic fibrosis gene, 280 kb (28); the factor VIII gene, 186 kb (29); the thyroglobulin gene, 100 kb (31); the insulin receptor gene, 120 kb (30); the cystic fibrosis gene, 280 kb (28); the factor VIII gene, 186 kb (29); the thyroglobulin gene, 100 kb (31); the insulin receptor gene, 120 kb (30); and the ornithine transcarbamylase gene, 78 kb (33)). Since the sum of the length is 1.9 kb, exons occupy only 2% of the entire gene, which is one of the lowest among genes heretofore reported. Although genes containing large introns are common, there does not appear to be a direct correlation between mRNA size and gene structure. For example, the human factor VIII (29) and thyroglobulin (30) genes exceed 100 kb, although these genes code for mRNA transcripts of several hundred bases, thereby eliminating exon 14, in this particular patient. A major abnormality in gene structure was not evident in the other patients studied.

human genome (28, 36, 37), the 5' side of intron 9, which we could not analyze, may locate within such a region.

The 5' end of the prolidase mRNA assigned here is 131 bases upstream from the initiation codon. The 5'-flanking region of the prolidase gene resembles that of other so-called house-keeping genes (38). It has a typical CAAT box and nuclease S1 mapping (B). See "Experimental Procedures" for details. Poly(A)" RNA from human placenta was analyzed. A, the primer was a synthetic oligonucleotide (20-mer, complementary to positions +114 to +133 (5' - ATGTTGCCGCCGCACCGGC-3')) 32P-labeled at the 5' end with [γ-32P]ATP and T4 polynucleotide kinase. Annealing was carried out with 0.35 pmol of the primer (3 x 106 dpm) and 8 μg of the poly(A)" RNA, and the mixture was subjected to reverse transcription. For a direct comparison with the genomic nucleotide sequence, the 5' ends of the probe and of the primer were arranged to coincide with the 5' ends of primers of the dioxygenase nucleotide sequencing (lanes G, A, T, and C). Portions of sample were electrophoresed in a 6% acrylamide, 7 M urea gel. Arrow indicates the position assigned to the 5' end of the mRNA. B, the probe was synthesized by polymerase chain reaction technique. A 5' primer (5'-CCTGCAGCCGCTCATTTATC-

Table II

| Restriction site | Size of fragment (kb) | Relative frequency (%) | Probe |
|------------------|----------------------|------------------------|-------|
| Kpn1             | +                    | 2.7                    | 0.2   | PL21 |
| -                | -                    | 23.0                   | 0.8   |      |
| BamHI            | +                    | 3.2                    | 0.25  | PL21 |
| -                | -                    | 3.4                    | 0.75  |      |
| EcoRV            | +                    | 1.0, 2.5               | 0.3   | pG14 |
| -                | -                    | 3.5                    | 0.7   |      |

For a 6.6-kb mRNA product and contains 25 introns (mean size 940 bp) spanning only 23 kb (34). The 15 exons contained in the prolidase gene range in size from 45 to 528 bp (mean size 135 bp), consistent with the documented distributions of exon size for 20 proteins (35). Fourteen introns of highly variable size separate the structure sequences into 15 exons. Intron 9 was over 50 kb, hence, we could not completely analyze it by three times of chromosome walking, and other human libraries were used. As unclonable regions are estimated to constitute 5% of the

- FIG. 3. Determination of the 5' end of human prolidase mRNA by primer extension (A) and nuclease S1 mapping (B). See "Experimental Procedures" for details. Poly(A)" RNA from human placenta was analyzed. A, the primer was a synthetic oligonucleotide (20-mer, complementary to positions +114 to +133 (5' - ATGTTGCCGCCGCACCGGC-3')) 32P-labeled at the 5' end with [γ-32P]ATP and T4 polynucleotide kinase. Annealing was carried out with 0.35 pmol of the primer (3 x 106 dpm) and 8 μg of the poly(A)" RNA, and the mixture was subjected to reverse transcription. For a direct comparison with the genomic nucleotide sequence, the 5' ends of the probe and of the primer were arranged to coincide with the 5' ends of primers of the dioxygenase nucleotide sequencing (lanes G, A, T, and C). Portions of sample were electrophoresed in a 6% acrylamide, 7 M urea gel. Arrow indicates the position assigned to the 5' end of the mRNA. B, the probe was synthesized by polymerase chain reaction technique. A 5' primer (5'-CCTGCAGCCGCTCATTTATC-
form of GGCGG), although it does not have a typical TATA box. Transcription factor Sp1 had been purified (39), and was shown to enhance transcription by RNA polymerase II 20-fold from several viral and cellular promoters that contained at least one properly positioned Sp1 binding site (39, 40). Transcription of the human prolidase gene may be responsive to the Sp1 factor. Multiple initiation sites for the transcription have been noted in several house-keeping genes (39).

Multiple initiation sites for the transcription of the gene when TaqI, BamHI, or EcoRI digested DNAs were used, and the cDNA insert of PL21 served as the probe. A partial structure of the gene and the probe used are shown below the gene structure. Lane 1, TaqI-digested DNA from the patient; lane 2, TaqI-digested DNA from the control; lane 3, BamHI-digested DNA from the control; lane 4, BamHI-digested DNA from the patient; lane 5, PstI-digested DNA from the control; lane 6, PstI-digested DNA from the patient.

RFLPs in the human prolidase gene were detected with the enzymes KpnI, BamHI, and EcoRV, the former two were responsive to the Spl factor. Multiple initiation sites for the transcription have been noted in several house-keeping genes lacking the TATA box (41–46). However, the initiation of prolidase mRNA transcription seems to occur at one site (Fig. 3).

RFLPs in the human prolidase gene were detected with the enzymes KpnI, BamHI, and EcoRV, the former two were detected with the cDNA insert of PL21 as a probe and the latter one was with a genomic fragment of pG14. The frequencies of the minor components of these three RFLPs are 0.2, 0.25, and 0.3, respectively (Table II). Locus of the prolidase gene was mapped to the short arm of the chromosome 19 (19p 10.1), as determined by linkage analysis (47), and the prolidase gene was reported to be close to the myotonic dystrophy locus, although some patients with prolidase deficiency are genetically heterogeneous, others seem to have different mutations.

Having characterized the normal gene for the human prolidase, further studies on the expression and regulation of this gene are underway. These studies will provide pertinent information required to analyze mutations and their effects in patients with prolidase deficiency.

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