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Molecular cloning of the α-subunit of human prolyl 4-hydroxylase: The complete cDNA-derived amino acid sequence and evidence for alternative splicing of RNA transcripts

(collagen/hydroxylases/hydroxyproline/proline)

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ABSTRACT **Prolyl 4-hydroxylase [procollagen-proline, 2-oxoglutarate 4-dioxygenase; procollagen-L-proline, 2-oxoglutarate:oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2], an $\alpha_2\beta_2$ tetramer, catalyzes the formation of 4-hydroxyproline in collagens by the hydroxylation of proline residues in peptide linkages. We report here on the isolation of cDNA clones encoding the α-subunit of the enzyme from human tumor HT-1080, placenta, and fibroblast cDNA libraries. Eight overlapping clones covering almost all of the corresponding 3000-nucleotide mRNA, including all the coding sequences, were characterized. These clones encode a polypeptide of 517 amino acid residues and a signal peptide of 17 amino acids. Previous characterization of cDNA clones for the β-subunit of prolyl 4-hydroxylase has indicated that its C terminus has the amino acid sequence Lys-Asp-Glu-Leu, which, it has been suggested, is necessary for the retention of a polypeptide within the lumen of the endoplasmic reticulum. The α-subunit does not have this C-terminal sequence, and thus one function of the β-subunit in the prolyl 4-hydroxylase tetramer appears to be to retain the enzyme within this cell organelle. Interestingly, three of the cDNA clones for the α-subunit contained a 64-nucleotide sequence homologous but not identical to the corresponding 64-nucleotide sequence found in four other cDNA clones. Nuclease S1 mapping experiments demonstrated that this difference was due to the existence of two types of mRNA present in approximately equal amounts. Southern blot analyses of human genomic DNA with a cDNA probe for the α-subunit suggested the presence of only one gene encoding the two types of mRNA, which appear to result from mutually exclusive alternative splicing of primary transcripts of one gene.**

Prolyl 4-hydroxylase [procollagen-proline, 2-oxoglutarate 4-dioxygenase; procollagen-L-proline, 2-oxoglutarate:oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2] catalyzes the formation of 4-hydroxyproline in collagens by the hydroxylation of proline residues in peptide linkages. This enzyme plays a central role in collagen synthesis, as the 4-hydroxyproline residues formed in the reaction are essential for the folding of the newly synthesized procollagen polypeptide chains into triple-helical molecules. The active prolyl 4-hydroxylase is a tetramer ($\alpha_2\beta_2$) with a molecular weight of ≈240,000 and consisting of two different types of enzymically inactive monomer with molecular weights of ≈64,000 (α-subunit) and ≈60,000 (β-subunit) (for a recent review, see ref. 1). Complete cDNA-derived amino acid sequences have recently been determined for the β-subunit of human (2) and chicken (3, 4) prolyl 4-hydroxylases. Surprisingly, this β-subunit has been found to be identical to the enzyme protein disulfide isomerase (2, 5, 6) and a major cellular thyroid hormone binding protein (7, 8) and highly similar to a glycosylation site binding protein of oligosaccharyl transferase (9).

The α-subunit of prolyl 4-hydroxylase probably contributes a major part of the catalytic site of the enzyme, and this subunit also appears to be regulated more efficiently than the β-subunit in response to changes in the rate of collagen synthesis (1). No amino acid sequence data have been reported for the α-subunit from any source, however. To obtain further information on the structure, biosynthesis, and regulation of prolyl 4-hydroxylase, we isolated cDNA clones for the α-subunit of human prolyl 4-hydroxylase and determined the complete cDNA-derived amino acid sequence for it.†

MATERIALS AND METHODS

Protein Purification and Amino Acid Sequence Determination. Prolyl 4-hydroxylase was isolated from human placentas (see ref. 10), and 25 μg of the enzyme was fractionated by 8% SDS/PAGE. The separated subunits were blotted onto a trichloroxy-(3-aminopropyl)silane-activated glass-fiber filter (11), and the proteins were detected by staining with 3,3-dipentyloxacarbocyanine iodide. The band corresponding to the α-subunit was cut out and subjected directly to automated Edman degradation (11) with an Applied Biosystems model 477A on-line 120A liquid-pulsed sequencer using narrow-bore reversed-phase analysis (Department of Medical Chemistry, University of Helsinki). Additional amino acid sequences were determined by preparing *Staphylococcus aureus* V8 protease peptides from the purified α-subunit as described (2).

Isolation of cDNA Clones. A plasmid cDNA library of the human tumor cell line HT-1080 (12) was screened with two 17-mer oligonucleotide mixtures radioactively labeled at their 5′ ends with T4 polynucleotide kinase and 5′-[γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). These oligonucleotide mixtures A (5′-CC$\overset{A}{G}$TA$\overset{A}{G}$TTNGCNACTTG-3′) and B (5′-CC$\overset{A}{G}$TA$\overset{A}{G}$TTNGCNACCTG-3′) corresponded to the amino acid sequence Gln-Val-Ala-Asn-Tyr-Gly of one V8 protease peptide. Duplicate library filters (13) were hybridized with 12.8 ng of oligonucleotide mixture per ml in 35% (vol/vol) formamide/6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 6.8)/1% (wt/vol) bovine serum albumin/1% (wt/vol) Ficoll/1% (wt/vol) polyvinylpyrrolidone/0.250 mg of denatured salmon sperm DNA per ml/0.1% (wt/vol) SDS at 37°C for 20 hr. The filters were washed with 2× SSC and 0.05% SDS first at 21°C and then at 40°C. One

Abbreviation: nt, nucleotide(s).

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M24486 and M24487).

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positive clone, HTA-2, was obtained. A 36-mer oligonucleotide derived from the HTA-2 sequence (nucleotides 1430–1465 of the α-subunit cDNA sequence) was used to screen a human placenta λgt11 cDNA library (Clontech) plated on a lawn of *Escherichia coli* Y1090. Duplicate copies on nitrocellulose filters were prepared (13) and hybridized with 1 ng of 5′-labeled oligonucleotide per ml as described above. Two positive recombinant phages, PA-11 and PA-15, were isolated and the inserts were ligated into the *Eco*RI site of plasmid pBR322 (13). To obtain additional cDNA clones, human placenta and fibroblast λgt11 libraries (Clontech) were first screened with ^{32}P-labeled nick-translated PA-11 and PA-15 full-length inserts and then with a 366-nucleotide (nt) 5′ *Eco*RI/*Hin*dIII fragment of PA-15 under stringent conditions.

Nucleotide Sequencing and Sequence Analysis. The nucleotide sequences were determined by the dideoxynucleotide sequencing method (14) using the Sequenase enzyme (United States Biochemical). The sequencing primers were either vector-specific primers or, in most cases, specific 17-mer oligonucleotides synthesized in an Applied Biosystems DNA synthesizer (Department of Biochemistry, University of Oulu). Sequence data were analyzed by the IBI/Pustell DNA and protein sequence analysis system. Homology comparisons with the National Biomedical Research Foundation Protein Data Bank and GenBank sequences were performed with Microgenie sequence software (Beckman).

Nuclease S1 Protection Analysis. To map the internal variable regions of the mRNAs, PA-52 and PA-15 were digested with *Hin*dIII, 3′-end-labeled by addition of [α-^{32}P]dGTP (3000 Ci/mmol) using the Klenow fragment of DNA polymerase I, and digested with *Bgl* II. The 3′-end-labeled 518-nt *Hin*dIII/*Bgl* II fragments of interest were isolated from a nondenaturing 5% polyacrylamide gel. Ten micrograms of human skin fibroblast poly(A)$^+$ RNA and 15,000 cpm of the 3′-end-labeled probes were used for nuclease S1 digestion at pH 4.8, 4.6, or 4.5, and the digestion products were examined by electrophoresis on a 5% polyacrylamide sequencing gel (15). Single-stranded DNA was prepared from the PA-52-derived 3′-end-labeled *Hin*dIII/*Bgl* II fragment (15) and was used for the nuclease S1 digestion at pH 4.8 as described above.

Transfer Blot Analysis of Poly(A)$^+$ RNA and Genomic DNA. Electrophoresis of human skin fibroblast poly(A)$^+$ RNA was performed in a 0.7% agarose gel containing 2 M formaldehyde, and the RNA was transferred to a nitrocellulose filter and hybridized (2, 13).

High molecular weight genomic DNA was isolated (13) from cultured human skin fibroblasts and digested completely with either *Bam*HI, *Eco*RI, or *Hin*dIII. The digested DNAs were fractionated electrophoretically on a 0.8% agarose gel, and the DNA was transferred to a nitrocellulose filter and hybridized (13).

RESULTS

Isolation of cDNA Clones. The amino acid sequences were determined for the N-terminal end of the prolyl 4-hydroxylase α-subunit and one *S. aureus* V8 protease peptide. A human tumor HT-1080 plasmid cDNA library consisting of 10^5 recombinants was screened with an oligonucleotide mixture prepared according to part of the V8 protease peptide sequence. The cDNA-derived amino acid sequence of one positive clone, HTA-2 (Fig. 1), fully matched this peptide sequence. Two longer overlapping clones, PA-11 and PA-15 (Fig. 1), were obtained by screening 2×10^5 phage recombinants of a human placenta λgt11 cDNA library using a 36-mer oligonucleotide derived from the HTA-2 sequence as a probe. Thirteen additional clones were obtained among 6×10^5 recombinants by rescreening the placenta cDNA library using as probes PA-11, PA-15, and a 366-nt 5′ *Eco*RI/*Hin*dIII fragment of PA-15 (to select for cDNA clones extending to the 5′ end of the corresponding mRNA). Four of these clones—PA-49, PA-52, PA-58, and PA-59 (Fig. 1)—were characterized further. The cDNA clones PA-11 and PA-15 and the 366-nt 5′ *Eco*RI/*Hin*dIII fragment of PA-15 were also used to screen 2×10^5 phage recombinants of a human fibroblast λgt11 library. Three positive clones were obtained, and one of them, FA-34 (Fig. 1), was characterized further.

Comparison of cDNA-Derived Amino Acid Sequences with Protein Sequences and the Amino Acid Composition of the α-Subunit. The sequence of 14-amino acid residues from the N-terminal end of the α-subunit matched the predicted sequence of the cDNAs with one exception (Fig. 2). The extreme N-terminal amino acid that remained ambiguous by protein sequencing, being either histidine or serine, was shown by nucleotide sequencing to be histidine. The N-terminal amino acid sequence for the V8 protease digestion peptide gave a precise match with the cDNA-derived amino acid residues 398–414 (Fig. 2). Furthermore, the amino acid composition calculated from the cDNA-derived amino acid sequence corresponded well with that reported for the rat (16) and chicken (17) prolyl 4-hydroxylase α-subunits (Table 1).

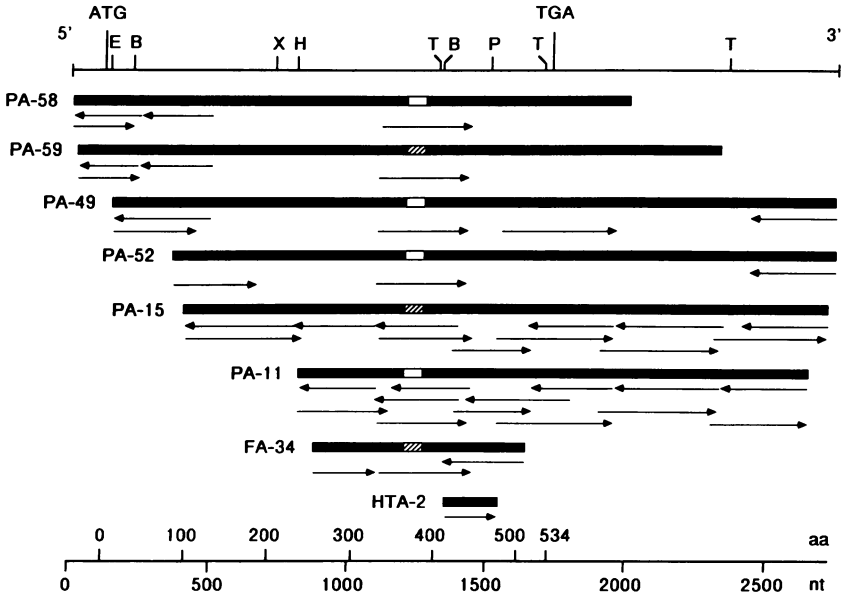

FIG. 1. Restriction maps and sequencing strategies for eight cDNA clones. The strategy for sequencing is indicated with arrows representing the directions and lengths of the sequencing runs. A partial restriction endonuclease map is given (B, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; T, *Taq* I; X, *Xba* I). □, A stretch of 64 nt present in clones PA-11, PA-49, PA-52, and PA-58; ▭, a stretch of 64 nt present in clones PA-15, PA-59, and FA-34. ATG indicates the beginning of translation and TGA is the end. nt and amino acid (aa) scales are shown at the bottom.

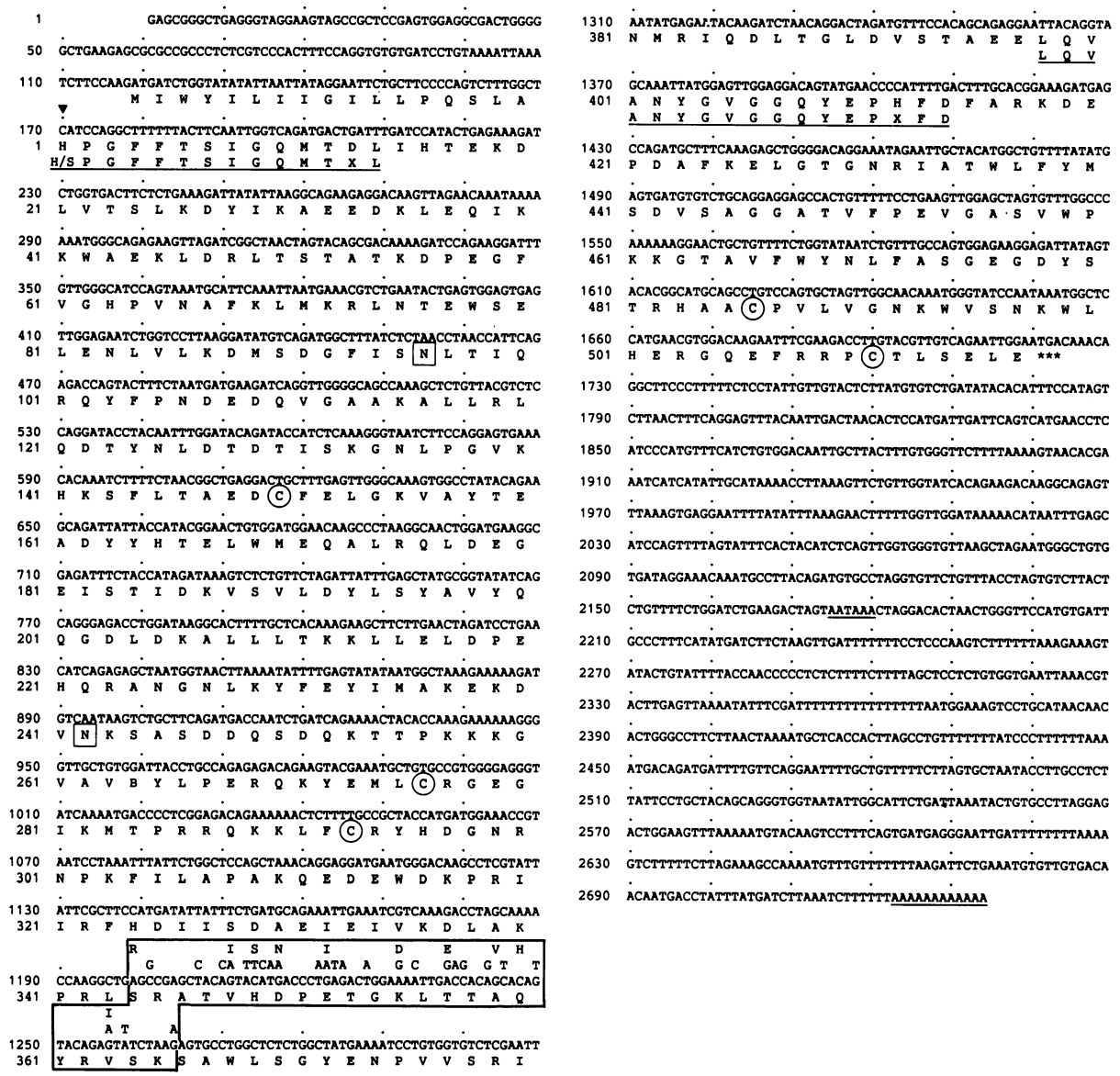

FIG. 2. Nucleotide and deduced amino acid sequences for cDNAs for the human prolyl 4-hydroxylase α-subunit. The amino acid sequences are shown by the one-letter code. ▼, N-terminal end of the processed α-subunit; ***, stop codon of translation. First continuous line, nucleotide sequence of cDNA clones HTA-2, PA-11, PA-49, PA-52, and PA-58. The nucleotides that are different within a 64-nt sequence and the predicted amino acid residues that change as a result of these nucleotide differences as being coded by clones PA-15, PA-59, and FA-34 are shown above the corresponding sequences in the other clones (nt 1199–1264, which include the variable region and the 2 nt preceding the first differing nucleotides, are boxed). The amino acid residues obtained by sequencing of the N-terminal end and one *S. aureus* V8 protease peptide of the α-subunit are shown below the continuous amino acid sequence and underlined. A polyadenylylation signal of AATAAA is also underlined. The cysteine residues are circled and the asparagine residues, which may serve as attachment sites for oligosaccharides, are boxed. The dC tails of the cDNA clone HTA-2 and the *Eco*RI linkers of all the other cDNA clones introduced to them during cloning are omitted. Numbering of the nucleotides begins with the extreme 5′ nucleotide of the α-subunit cDNA clones and dots indicate every 10th nucleotide. Numbering of the amino acid residues begins with the first residue of the processed α-subunit.

Nucleotide and Derived Amino Acid Sequences of the cDNAs. The cDNA clones encode a 534-amino acid polypeptide (Fig. 2). A hydrophobic 17-amino acid residue sequence beginning with methionine, presumably the signal peptide, precedes the N-terminal end of the α-subunit obtained by protein sequencing. The molecular weight of the polypeptide, excluding the signal sequence, is 59,000 and the predicted pI is 6.15. The cDNA-derived amino acid sequence contains one sequence of -Asn-Leu-Thr- and one of -Asn-Lys-Ser- (Fig. 2), which may serve as attachment sites for asparagine-linked oligosaccharides. No C-terminal Lys-Asp-Glu-Leu sequence was found (see *Discussion*). The hydrophilicity/hydrophobicity plot indicates that the polypeptide is predominantly hydrophilic, especially in its central region (data not shown).

The cDNA clones cover 118 nt of the 5′ and 1001 nt of the 3′ untranslated sequences (Fig. 2). The extreme 3′ untranslated region contains a 12-nt poly(A) sequence, which is not preceded by a canonical polyadenylylation signal. Therefore, this stretch is unlikely to represent the poly(A) tail. A polyadenylylation signal AATAAA is found 541 nt 5′ of the 3′ end of the cDNA sequence, but no corresponding mRNA species were detected in a Northern blot (see Fig. 4). No homologies were found when the nucleotide and predicted amino acid sequences were compared with the GenBank nucleotide and National Biomedical Research Foundation protein data base sequences.

Alternative Splicing of the α-Subunit RNA Transcripts. Comparison of the eight overlapping cDNA clones indicated that

Table 1. Comparison of the cDNA-derived amino acid composition of the α-subunit of human prolyl 4-hydroxylase with those reported for the α-subunit of rat and chicken prolyl 4-hydroxylase

Amino acid	Residues per 517 amino acids		
	Rat subunit	Chicken subunit	Human subunit
Asn			19
Asp	52*	54*	40
Thr	29	32	32
Ser	27	34	28
Gln			21
Glu	65*	65*	41
Pro	21	19	21
Cys	4	8	5
Gly	39	42	32
Ala	38	37	36
Val	28	26	29
Met	13	9	9
Ile	21	18	21
Leu	54	55	50
Tyr	22	23	21
Phe	18	19	20
His	13	10	12
Lys	44	47	45
Arg	29	31	25
Trp	ND	ND	10

Rat data are from ref. 16; chicken data are from ref. 17; human data were determined from the cDNA-derived amino acid sequence. ND, not determined.
*Asn + Asp or Gln + Glu.

three of them—PA-15, PA-59, and FA-34—contain a 64-nt sequence that replaces a sequence of identical length in four other cDNA clones (Figs. 1 and 2). One of the clones, HTA-2, did not cover this region. This sequence difference does not alter the reading frame. The two 64-nt sequences are 61% homologous at the nucleotide level and 55% homologous at the amino acid level (68% homologous if conservative amino acid changes are included). No other differences were detected in the nucleotide sequences of the overlapping clones.

To determine whether the differences in the cDNA clones reflected differences in the respective mRNAs, the region concerned was analyzed by nuclease S1 mapping of the RNAs. Two double-stranded cDNA probes were prepared that were otherwise identical except for a difference of 64 nt (Fig. 3*A*). When poly(A)$^+$ RNA from human skin fibroblasts was hybridized with the two probes and then subjected to nuclease S1 digestion at pH 4.8, bands of 510 and 390 nt were observed (Fig. 3*B*). With both probes, the more intense 510-nt band represented the fully protected cDNA probe and the partially protected fragments of 390 nt were of the size to be expected if some of the mRNA species contained the 64-nt sequence found alternatively in the cDNA clones. Lowering the pH of the nuclease S1 digestion mixture to 4.6 or 4.5 resulted in the disappearance of both the fully and partially protected fragments (data not shown). Apparently at a pH closer to the optimum (pH 4.3) of nuclease S1, the enzyme began to digest the hybrids to such an extent that the ^{32}P label at the 3′ ends of the probes was lost. If the two mRNA species corresponding to clones PA-52 and PA-15 exist in equal amounts, the ratio of fully to partially protected DNA probe is expected to be equal. The second possibility is that one of the mRNA species is more predominant than the other. In that case, the fully protected band should be prominent with one of the probes, while the partially protected band should be prominent with the other. Neither of these results was obtained, however.

To clarify whether the intense fully protected bands were DNA·DNA hybrids instead of DNA·RNA hybrids owing to

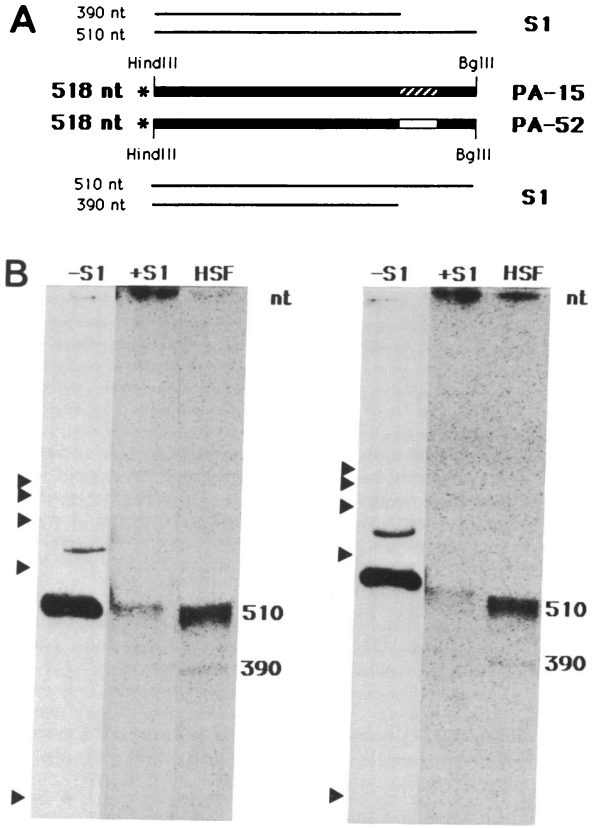

FIG. 3. Nuclease S1 mapping of poly(A)$^+$ RNA with cDNA clones PA-15 and PA-52. (*A*) Two 518-nt *Hin*dIII/*Bgl* II probes were prepared from the overlapping clones PA-15 and PA-52 and labeled (*) at their 3′ ends. A stretch of 64 nt (▨) in the PA-15-derived probe is different from the identical length sequence (▭) in the PA-52-derived probe. The nuclease S1 digestion products obtained with the PA-15-derived probe are shown above the probe and those obtained with the PA-52-derived probe are shown below the probe. (*B*) Autoradiography of the nuclease S1 digestion products fractionated by gel electrophoresis. Migration (from top to bottom) of bacterial phage φX174 *Hae* III fragments (1353, 1078, 872, 603, and 310 nt) is indicated by arrowheads. Lanes: −S1, probe without nuclease S1; +S1, probe with nuclease S1 in the absence of RNA; HSF, products of nuclease S1 digestion of hybrids of the 3′-end-labeled probes and 10 μg of poly(A)$^+$ RNA from human skin fibroblasts. (*Left*) Experiment using the PA-15-derived probe. (*Right*) Experiment with the PA-52-derived probe.

the use of double-stranded probes, a single-stranded probe was prepared from the above PA-52-derived probe. The same ratio of the fully to partially protected DNA probe was obtained with the single-stranded probe (data not shown) as obtained with the two double-stranded probes. This suggests that nuclease S1 does not digest mismatched hybrids to completion, probably because of the homology of the two 64-nt sequences. Therefore, taking into account that approximately equal numbers of both types of cDNA clones were obtained and that identical results were obtained with the two double-stranded probes in the nuclease S1 protection experiments, it seems likely that the two mRNAs exist in approximately equal amounts in human skin fibroblasts.

Northern and Southern Transfer Blot Analysis. The mRNA hybridizing with the clones encoding the α-subunit was 3000 nt long (Fig. 4, lane A). To obtain information on the gene(s) encoding the α-subunit, genomic DNA was digested with *Bam*HI, *Eco*RI, and *Hin*dIII, and the products were analyzed by Southern blot hybridization. A 657-nt *Taq* I/*Taq* I fragment of clone PA-11 that corresponds to the end of translated sequences and over half of the 3′ untranslated sequences was used as a probe. A single 16.5-kilobase (kb) fragment was

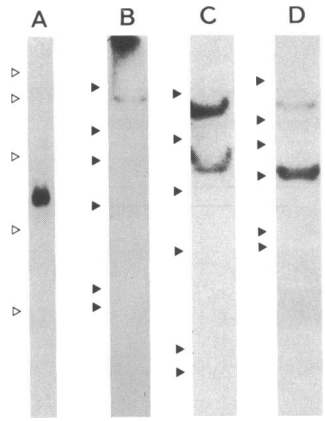

FIG. 4. Transfer blot analysis of poly(A)$^+$ RNA and genomic DNA. Lane A, 10 μg of human skin fibroblast RNA hybridized with the ^{32}P-labeled nick-translated cDNA clone PA-49. Migration (from top to bottom) of RNA markers (9.5, 7.5, 4.4, 2.4, and 1.4 kb) is indicated by open arrowheads. Lanes B, C, and D, *Bam*HI, *Eco*RI, and *Hin*dIII digests, respectively, of human genomic DNA hybridized with a ^{32}P-labeled 657-nt *Taq* I/*Taq* I fragment of clone PA-11 covering nt 1689–2345. Migration (from top to bottom) of *Hin*dIII fragments of λ phage DNA (23, 9.4, 6.6, 4.3, 2.3, and 2.0 kb) is indicated by solid arrowheads.

seen in the *Bam*HI digest, while two fragments of 15.5 and 7.7 kb were detected in the *Eco*RI digest and two fragments of 12.5 and 4.4 kb were seen in the *Hin*dIII digest (Fig. 4, lanes B–D). This pattern suggests that a single gene encodes the α-subunit of prolyl 4-hydroxylase.

DISCUSSION

The data reported here indicate that the catalytically important α-subunit of prolyl 4-hydroxylase consists of 517 amino acid residues and a signal peptide of 17 amino acids. The cDNA-derived amino acid sequence was unique among the currently available data bank sequences, but as no cloning or amino acid sequencing data are yet available for the other 2-oxoglutarate dioxygenases (see ref. 1), subsequent research may point to protein sequences homologous to those of the α-subunit.

It has been proposed that proteins functioning within the lumen of the endoplasmic reticulum possess a C-terminal sequence Lys-Asp-Glu-Leu, which may be both necessary and sufficient for the retention of a polypeptide within this cell organelle (18). Prolyl 4-hydroxylase is located within the cisternae of the rough endoplasmic reticulum (1) and the multifunctional β-subunit of the enzyme tetramer has been found to possess this C terminus in all the species studied so far (2–5, 8). The present data indicate that the α-subunit does not have this C-terminal sequence, and thus one function of the β-subunit in the prolyl 4-hydroxylase tetramer may be that of retaining the enzyme within the lumen of the rough endoplasmic reticulum.

An interesting feature of the α-subunit is that its RNA transcripts were found to undergo alternative splicing. Two types of cDNA clone were identified that differ over a stretch of 64 nt, and the nuclease S1 mapping experiments demonstrated that normal human skin fibroblasts contain mRNAs corresponding to both types of cDNA. Southern blot analyses of human genomic DNA with a cDNA probe for the α-subunit suggested the presence of only one gene encoding this subunit. This suggestion was supported by the finding that no other differences were detected in the nucleotide sequences of the overlapping clones including the 5′ and 3′ untranslated sequences. The two types of mRNA thus probably result from alternative splicing of the primary transcripts of one gene. Since no cDNA clone was found to contain or lack both 64-nt stretches, these sequences appear to be present in a mutually exclusive fashion (19). The biological significance of this finding is currently unknown.

Two forms of α-subunit have previously been found in prolyl 4-hydroxylase from many sources (20). In chicken embryo fibroblasts, one form contains two asparagine-linked high mannose oligosaccharides, while the other form contains only one (20). It has been suggested that these two forms represent two highly similar but distinct polypeptides in which slight differences in amino acid sequence may result in the use of two glycosylation sites on one form but only one on the other (20). The present data indicate that the human α-subunit contains two potential attachment sites for asparagine-linked oligosaccharides, an -Asn-Leu-Thr- sequence and an -Asn-Lys-Ser- sequence. Neither of these is located in the differentially spliced segment of the human α-subunit, however, and the relation between the two differently glycosylated forms of the chicken α-subunit and the alternative splicing of the primary RNA transcripts reported here thus remains unknown.

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