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Structure of a full-length cDNA clone for the preproα2(I) chain of human type I procollagen

Comparison with the chicken gene confirms unusual patterns of gene conservation

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A cDNA clone from a human placental library was found to consist of an essentially full-length cDNA of 4.6 kb for the preproα2(I) chain of type I procollagen. Nucleotide sequencing of the 5'-end of the cDNA provided a sequence of 1617 nucleotide residues and codons for 539 amino acid residues not previously defined. Comparison of the complete structure of the preproα2(I) cDNA with previously reported sequences for the chicken proα2(I) gene indicated that 83% of 1366 total amino acid residues were conserved. In the α-chain domain 84% of 1014 amino acid residues were conserved. Also, there was conservation of the previously noted preference for U and C in the third position of codons for glycine, proline and alanine. One major difference between the human and the chicken preproα2(I) chain was that the human chain contained 21 fewer proline residues, an observation that probably explains why the triple helix of human type I procollagen unfolds at temperatures that are 1–2 °C lower. In parallel experiments, sequencing of intron–exon boundaries for nine exons of genomic subclones confirmed and extended previous observations that the proα2(I) gene, like other genes from fibrillar collagens, has an unusual 54-base pattern of exon sizes that is highly conserved through evolution.

INTRODUCTION

Type I collagen provides the fibrous network that maintains the structural integrity of most tissues in vertebrates and in many other multicellular organisms (for reviews see Prockop & Kivirikko, 1984; Cheah, 1985). The protein is a heterotrimer of two α1(I) and one α2(I) chains, and it is first synthesized as a procollagen comprised of two proα1(I) and one proα2(I) chains. Large parts of the primary structure of the α1(I) and α2(I) chains of type I collagen are now known. Most of the initial data were generated by Edman degradation of peptide fragments (see Kang *et al.*, 1967; Fietzek *et al.*, 1972; Piez, 1976; Dixit *et al.*, 1978; Hofmann *et al.*, 1978; Galloway, 1982), but, because analysis of the protein is far more difficult, most of the recent data were derived from nucleotide sequencing of DNA clones (Bernard *et al.*, 1983*a*,*b*; Boedtker *et al.*, 1985; Dickson *et al.*, 1985). However, it has been difficult to obtain a complete amino acid sequence for an α(I) or proα(I) chain from a single species. The most complete sequence came from the analysis of a combination of cDNA and genomic clones for the proα2(I) chain from chicken, an analysis that lacks only the coding sequences of two exons that contain 36 codons (Boedtker *et al.*, 1985). Also, apparently because of the high G+C content of the relatively long mRNAs, no full-length cDNA is available for a proα1(I) or proα2(I) chain of type I procollagen.

In the present paper we describe the first full-length cDNA clone coding for a preproα2(I) chain. Nucleotide sequencing of the cDNA has permitted detailed comparison of evolutionary differences between the human and chicken procollagen gene. Also, since the full-length cDNA developed here is for the human preproα2(I) chain, the clone provides both a probe and structural information that will be of great use in current attempts to define mutations in type I procollagen genes that produce heritable disorders of connective tissue such as osteogenesis imperfecta and Ehlers–Danlos syndrome (see Prockop & Kivirikko, 1984; Byers & Bonadio, 1985; Prockop & Kuivaniemi, 1986).

In the text of the present paper amino acid positions are numbered by the standard convention in which the first glycine residue of the triple-helical domain of an α chain is number 1. The numbers for the α2(I) chain can be converted into those of the human preproα2(I) chain in Fig. 1 by adding 90.

Preliminary reports of this work were presented at the East Coast Connective Tissue Society Meeting, Wood Hole, MA, U.S.A., in March 1987.

MATERIALS AND METHODS

cDNA and genomic clones

A cDNA library from human placenta was obtained from a commercial source (catalogue no. HL 1008; Clontech, Palo Alto, CA, U.S.A.). The library was

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These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00724.

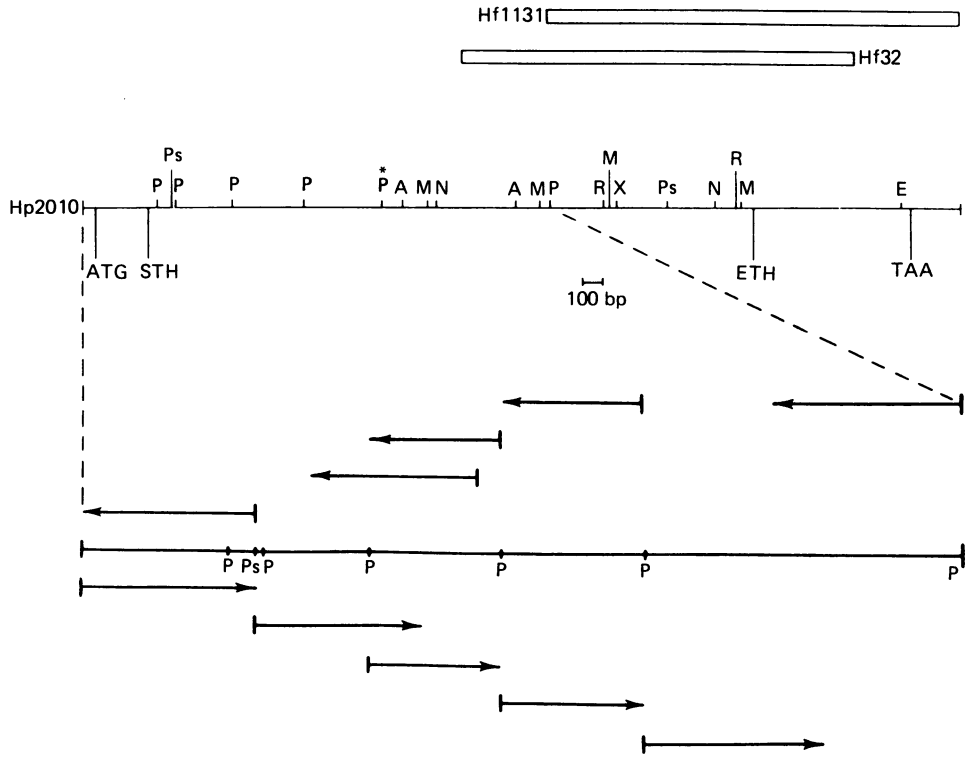


Fig. 1. Partial restriction map of the cloned cDNA for the preproα2(I) chain (Hp 2010)

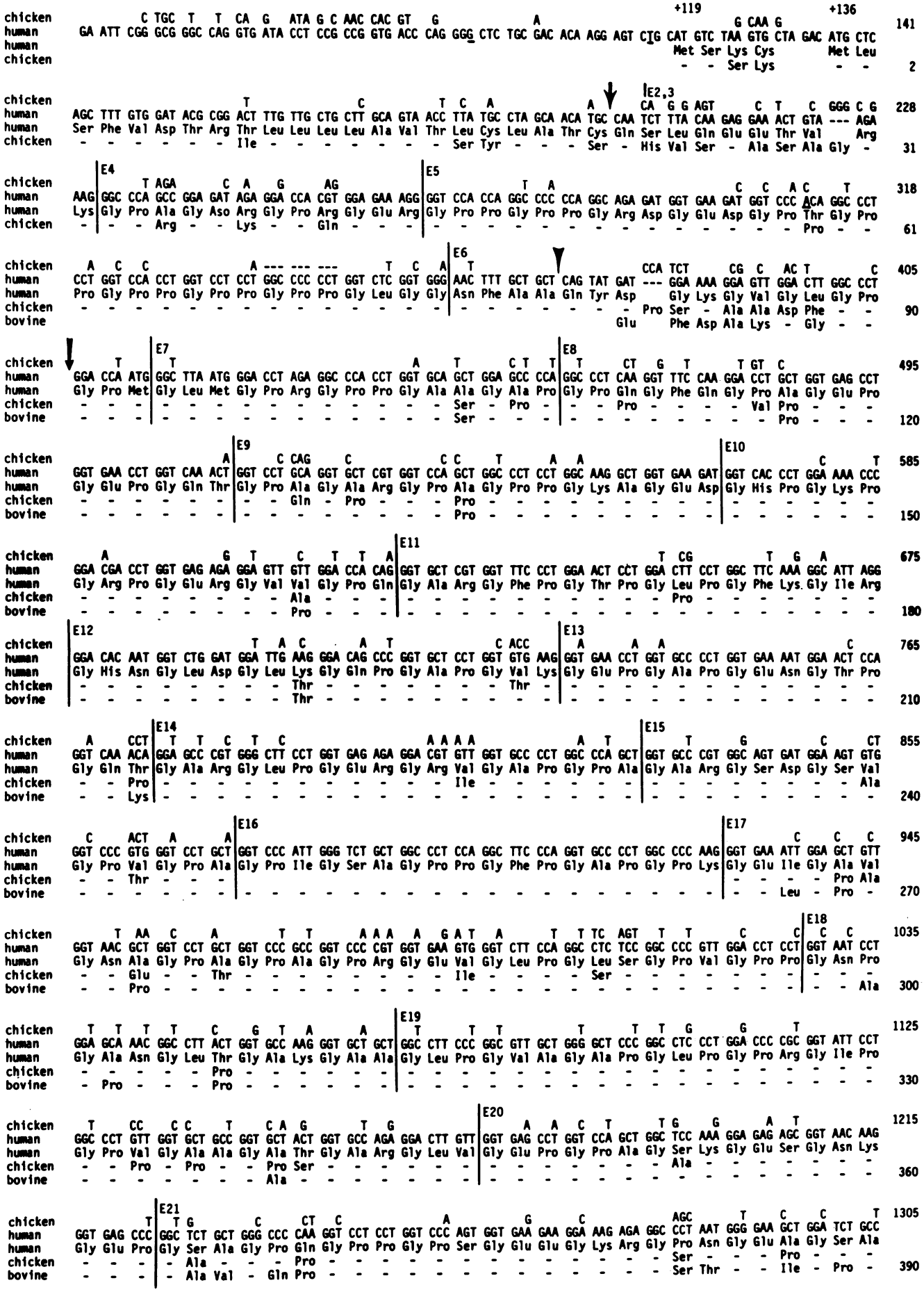
Also shown, the relative sizes of two clones previously reported (Bernard *et al.*, 1983*a*) for the human proα2(I) chain (Hf-1131 and Hf-32). Symbols: ATG, start site of translation; STH, start of the triple-helical domain; ETH, end of triple-helical domain; TAA, end of translation; A, *Ava*I site; E, *Eco*RI site; M, *Mst*II site; N, *Nco*I site; P, *Pvu*II site; Ps, *Pst*I site; R, *Rsa*I site; X, *Xho*I site. The asterisk indicates a variable *Pvu*II site that was found in Hp-2010 and in the two clones of genomic allele sequenced here, but not in a short cDNA clone for the proα2 chain (Hf-15; M.-L. Chu & M. Bernard, unpublished work). As noted in the text, regions not sequenced in both directions were confirmed by analysis of genomic subclones.

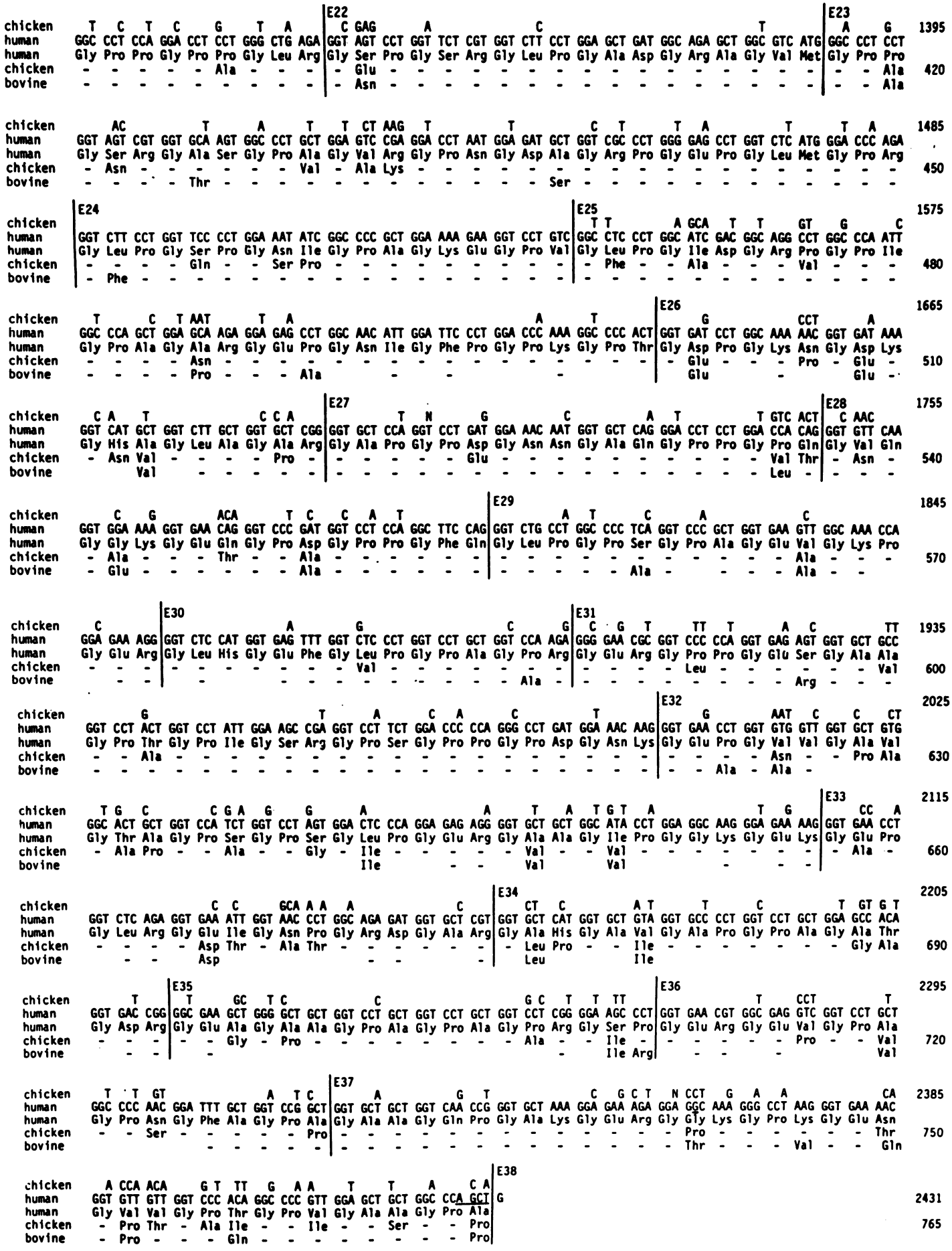
prepared with cDNA that was not size-selected, that was inserted into the bacteriophage vector λgt11, and that was amplified before distribution. It contained 10^6 independent clones with an average size of 1.8 kb and a range of sizes of 0.8–3.6 kb. The probe used to screen the library was a 2 kb *Hin*dIII–*Hin*dIII fragment that was a subclone of the proα2(I) genomic DNA obtained from a human bacteriophage library (clone NJ-3; Myers *et al.*, 1983). The 2 kb *Hin*dIII–*Hin*dIII fragment was subcloned into plasmid pBR322 from clone NJ-3 by Mr. Bruce Vogel.

One series of genomic subclones contained exons 10–12 of the proα2(I) gene and the other contained exons 25–30. The first series was prepared with 10 kb *Eco*RI–*Eco*RI fragments of genomic DNA from a proband with atypical Ehlers–Danlos syndrome (Sippola *et al.*, 1984; de Wet *et al.*, 1986). The fragments were cloned into the bacteriophage vector Charon 4A, the clones were screened with a 2 kb *Hin*dIII–*Hin*dIII fragment from the proα2(I) genomic clone NJ-3, and a 2.3 kb *Bam*HI–*Eco*RI fragment was subcloned into M13 for nucleotide sequencing. The normal allele was dis-

Fig. 2. Nucleotide and amino acid sequence of the cDNA clone for the proα2(I) chain

The nucleotides are numbered from the start site for transcription (Dickson *et al.*, 1985) and the amino acids from the first amino acid residue of the preproα2(I) chain. The 2379 bp nucleotide sequences from the 5′-end of the cDNA clone are indicated in the second line. The overlap with previously published sequences from Hf-32 (Bernard *et al.*, 1983*a*) starts at position 2002. The first 7 bp shown here are from the *Eco*RI linker with which the clone was inserted into λgt11 vector. The amino acid sequence encoded for by the clone is indicated in the third line. Top line: nucleotide sequences for the chicken preproα2(I) chain where they are known and differ from the human sequence. Lines two and three: nucleotide and amino acid sequences of Hp-2010 defined here. Line four: amino acid sequences encoded for by the chicken preproα2(I) cDNA and genomic clones reported previously (Boedtker *et al.*, 1985). Line five: amino acid sequences of the bovine α2(I) chain that was defined by Edman degradation of peptide fragments (see Galloway, 1982). Symbols; -, identical amino acid; ---, missing nucleotide residues in the human or chicken cDNA; +119 a possible start site for translation that ends in a stop codon after 11 nucleotide residues; +136, start site for translation; vertical lines, beginnings of exons indicated; ▼, cleavage site for signal peptidase; ▼, cleavage site for procollagen N-proteinase; ▼, beginning of α chain domain. The coding sequences found in exon 16 from the chicken gene are not known. The amino acid residues encoded by exon 24 of the chicken gene are known by Edman degradation of peptide fragments, but the nucleotide residues are not known (see Galloway, 1982).

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2385
750
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765

tinguished from the abnormal allele by the presence of a 19 bp deletion in the abnormal allele that produced an abnormally spliced mRNA, whereas the normal allele produced mRNA that was normally spliced (H. Kuivaniemi, C. Sabol, G. Tromp, M. Sippola-Thiele & D. J. Prockop, unpublished work). The second series of genomic subclones was prepared with 6 kb *Hin*dIII–*Hin*dIII fragments of genomic DNA from a proband with a lethal variant of osteogenesis imperfecta (de Wet *et al.*, 1983, 1986). The fragments were cloned into the bacteriophage vector Charon 21A, and the clones were screened with a 3.6 kb *Hin*dIII–*Eco*RI fragment from the proα2(I) genomic clone NJ-3. A series of 3.6 kb *Hin*dIII–*Eco*RI fragments from positive clones were subcloned into M13 for nucleotide sequencing. The normal sequences for genomic DNA covering exons 25–30 and their flanking sequences were distinguished from the abnormal ones by the fact that the abnormal allele had a single base mutation and gave rise to an abnormally spliced mRNA (G. Tromp & D. J. Prockop, unpublished work).

Nucleotide sequencing of the cDNA and genomic clones

The cDNA library was first plated on superconfluent cultures with about 100000 clones on each of ten 15 cm-diameter Petri dishes. Twenty-three positive clones were plaque-purified. Fragments of Hp-2010 were subcloned into M13mp18 and M13mp19, and the nucleotide sequencing with the dideoxy method was carried out by using universal primers (Sanger *et al.*, 1977; Messing, 1983). Each fragment was sequenced at least three times. The sequences were first defined by using the Klenow fragment of DNA polymerase I, but most of the sequences were verified by using a modified T7 DNA polymerase (Sequenase, as supplied by U.S. Biochemical Corp.; Tabor & Richardson, 1987) and the dGTP analogue dITP. Use of the T7 DNA polymerase was essential to establish some of the sequences because of the high C+G content of the cDNA. The genomic subclones in M13 were sequenced with the same procedures except that the subclones containing exons 10–12 were sequenced first with primers specific for sequences in the exons and then with primers specific for adjacent regions of the intervening sequences (H. Kuivaniemi, C. Sabol, G. Tromp, M. Sippola-Thiele & D. J. Prockop, unpublished work).

Other procedures

All the DNA probes were labelled by nick-translation with [α^{32}P]dCTP. Standard procedures were used for Northern-blot analysis of total RNA from normal human skin fibroblasts and for Southern-blot analysis of genomic fragments.

RESULTS AND DISCUSSION

Restriction map and nucleotide sequence of the cDNA

The cDNA library from human placenta was screened with a 2 kb *Hin*dIII–*Hin*dIII fragment from the 5′-end of the human gene for the proα2(I) chain of type I procollagen (Myers *et al.*, 1983). Twenty-three positive clones were plaque-purified and the inserts were analysed by digestion with *Eco*RI. Four of the clones had inserts ranging from 3.6 to 4.8 kb. By Northern blot analysis, all four cDNAs hybridized (results not shown) to the mRNAs of three different sizes previously demonstrated to be specific for the proα2(I) chain of type I procollagen (Myers *et al.*, 1983). The 4.6 kb clone Hp-2010 was selected for detailed analysis. When used as a probe for Southern blot analysis, it hybridized to all the expected fragments from two genomic clones for the proα2(I) gene (NJ-1 and NJ-3 in Myers *et al.*, 1983; results not shown).

The 3′-end of the cDNA clone Hp-2010 had the same restriction-endonuclease map as previously reported cDNAs from the 3′-end of the proα2(I) gene (Bernard *et al.*, 1983*a*). These previously analysed cDNAs covered the codons for amino acid residues 533 to 1014 of the α-chain domain, the *C*-terminal telopeptide, all the 243 amino acid residues of the C-propeptide, and the 3′-non-translated region of the mRNA. In total, they included 2468 bp of the mRNA and codons for 740 amino acid residues. Here we determined 1617 nucleotide residues and the codons for 539 amino acid residues not previously defined (Figs. 1 and 2). In addition, we re-examined the sequence of 425 bp that overlapped the 5′-end of the previously published cDNA sequences (Bernard *et al.*, 1983*a*) and 327 bp defined by sequencing genomic clones containing exons 1, 4, 5 and 6 (Dickson *et al.*, 1985). As indicated in Fig. 1, over 80% of the base-pairs were sequenced in both directions. The regions not sequenced in both directions were confirmed by nucleotide sequencing of the corresponding exons in genomic subclones (see below).

The data revealed several minor differences from previously reported sequences. In regions analysed in cDNAs (Bernard *et al.*, 1983*a*) there was a single base difference that converted an alanine codon for amino acid position 653 of the α2(I) chain into a codon for glycine. In the sequenced exons from the 5′-end of the gene (Dickson *et al.*, 1985), there was a single base difference that converted a proline codon in amino acid position 59 of the propeptide into a codon for threonine. These two differences may or may not be variants in the gene structure. Also, the data defined a G and a T residue in the 5′-non-translated region that were previously ambiguous (Dickson *et al.*, 1985). In addition, the data suggested the possible presence of a new polymorphic site (Tsipouras *et al.*, 1983; Grobler-Rabie *et al.*, 1985; Sykes *et al.*, 1986) for cleavage of the gene by *Pvu*II in exon 25 (Fig. 1). The site was present in a previously isolated genomic clone for the proα2(I) gene (clone NJ-3; Myers *et al.*, 1983). It was present in subclones of both alleles from the proband with a lethal variant of osteogenesis imperfecta (de Wet *et al.*, 1983). It was found in the DNA clone Hp-2010. However, it was not found in Hf-15, a cDNA clone of about 0.8 kb that was isolated from a human skin fibroblast library (M.-L. Chu & M. Bernard, unpublished work).

Conservation of amino acid sequences

The data as a whole made it possible to make a detailed comparison (Fig. 2 and below) of the nucleotide and amino acid sequences of the human preproα2(I) chain and the chicken preproα2(I) chain (Boedtker *et al.*, 1985). About 83% of the 1366 of the amino acid residues in the total human chain were identical with the chicken

Table 1. Common amino acid substitutions between human and chicken preproα2(I) chains

Changes indicated account for 90 out of 227 amino acid substitutions

Amino acid in human chain	Amino acid in chicken chain				
	Ala	Ser	Pro	Val	Thr
Ala		4	18	6	2
Ser	5		2	1	4
Pro	8	3		3	2
Val	9	1	5		4
Thr	4	3	5	1	

chain. In the α-chain domain 84% of the 1014 amino acids residues were identical.

Most of the amino acid differences were conservative substitutions. Ninety (40%) of 227 differences in the preproα2(I) chains were accounted for by five residues: alanine, serine, proline, valine and threonine (Table 1). The most striking difference in amino acids between the human and the chicken chain was that the human chain contained 21 fewer proline residues in the triple-helical domain. Of these, 14 were in the Yaa-position and therefore are likely to be converted into 4-hydroxyproline during procollagen biosynthesis (see Prockop & Kivirikko, 1984). The terminal stability of the collagen triple helix depends in large part on its content of proline and hydroxproline residues. Therefore the difference in imino acids probably explains why the triple helix of the chicken type I procollagen unfolds with a T_m ('melting' temperature) of about 42 °C whereas human type I procollagen has a T_m that is 1–2 °C lower (Hayashi *et al.*, 1979; Peltonen *et al.*, 1980).

Conservation of codon usage between the human and chicken proα2(I) cDNAs

Previous reports indicated an unusual preference for U or C in the third base of codons for glycine, proline and alanine in cDNAs for human and chicken α1(I) and α2(I) chains (Bernard *et al.*, 1983*a*,*b*; Boedtker *et al.*, 1985). As indicated in Table 2, the same preference was largely maintained when the data for the whole human α2(I) chain were analysed. Of note was that there was a greater preference in the human α2(I) sequence for U in the third position of codons for proline that were in the Yaa-position of the repeating -Gly-Xaa-Yaa- sequence of the collagen α-helix than in the total proline codons. The preference for U in the third position of the Yaa-position proline codons is probably explained by the avoidance of

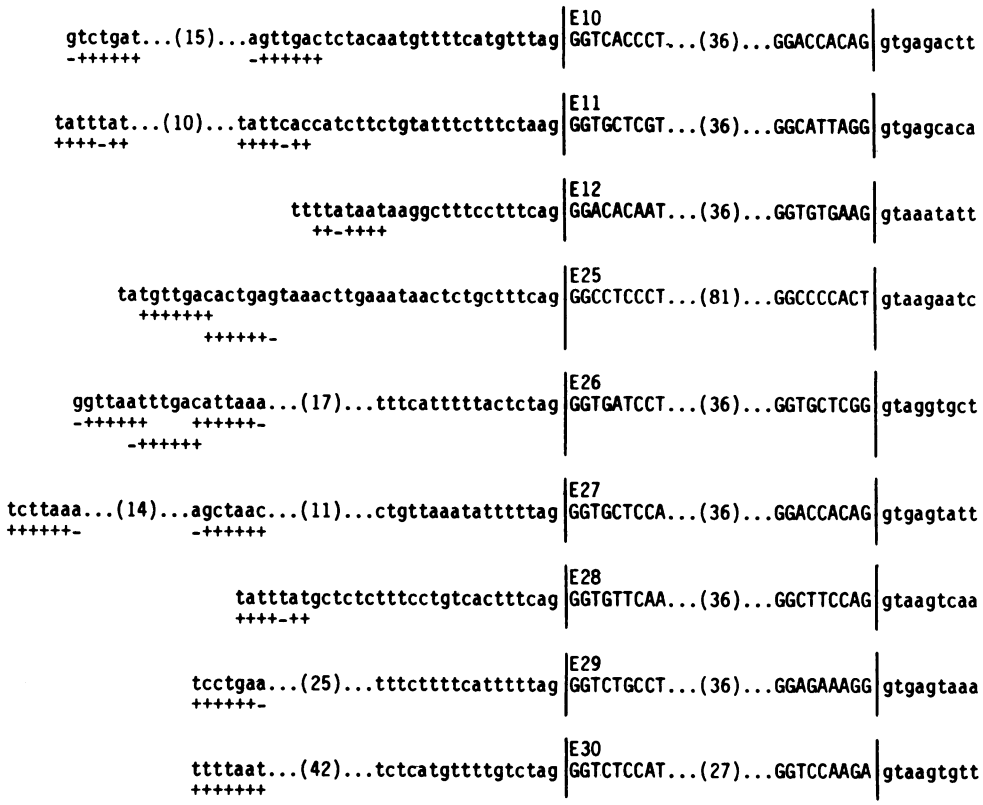

Fig. 3. Nucleotide sequences of the exon/intron boundaries of nine exons and the lariat-loop sites for the 5′ intervening sequences

Symbols: +, nucleotides that are identical with the consensus sequence; -, nucleotides that lack identity with the consensus sequence; numbers in parentheses, number of bases between those shown. The normal consensus sequence is PyNPyTPuAPy (Ruskin *et al.*, 1984).

Table 2. Codon usage in α2(I) domain of chicken and human type I procollagen

Data for the chicken α2(I) chain are from Boedtker *et al.* (1985). No nucleotide sequence data are available for exons 16 and 24 from the chicken gene. These exons contain 36 amino acid residues in the human proα2(I) gene and include ten proline, 12 glycine and three alanine residues (see Fig. 2).

Amino acid	Chick	Human	Third base
Gly	0.64	0.51	U
	0.18	0.22	C
	0.16	0.22	A
	0.02	0.05	G
Codons examined ...	330	342	
Pro (total)	0.70	0.62	U
	0.09	0.21	C
	0.20	0.16	A
	0.01	0.01	G
Codons examined ...	211	199	
Pro (Yaa-position)	0.81	0.73	U
	0.02	0.09	C
	0.17	0.16	A
	0	0.02	G
Codons examined ...	100	91	
Ala	0.82	0.76	U
	0.07	0.15	C
	0.10	0.08	A
	0	0	G
Codons examined ...	97	107	

C-G dinucleotide sequences (Bird, 1986; Brown & Bird, 1986) when the Yaa-position proline is followed by glycine (GGN).

Conservative 54 bp pattern in exons of proα2(I) gene

In parallel experiments, nucleotide sequencing was carried out on two series of genomic subclones for the human proα2(I) gene. Data were generated for nine exons not previously sequenced (Fig. 3). Seven of the exons were 54 bp, one was 45 bp and one was 99 bp. These exons were previously reported to have the same sizes in the chicken proα2(I) gene (Boedtker *et al.*, 1985). Therefore the results extend previous observations indicating that the proα2(I) gene, like other genes for fibrillar collagens (Ohkubo *et al.*, 1980; Vogeli *et al.*, 1980; Yamada *et al.*, 1980; Dickson *et al.*, 1985; Boedtker *et al.* 1985; Cheah, 1985), has an unusual 54 bp pattern of exon sizes and that the pattern is highly conserved through evolution. The data presented in Fig. 3 also provide the nucleotide sequences of the exon/intron boundaries and the sites for lariat-loop formation in nine of the intervening sequences. The boundary sequences and the sites for the lariat loop are, in general, homologous with similar sequences in other genes (Mount, 1982; Ruskin *et al.*, 1984; Reed & Maniatis, 1985; Padgett *et al.*, 1986).

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REFERENCES

Bernard, M. P., Myers, J. C., Chu, M.-L., Ramirez, F., Eikenberry, E. F. & Prockop, D. J. (1983*a*) Biochemistry **22**, 1139–1145
Bernard, M. P., Chu, M.-L., Myers, J. C., Ramirez, F., Eikenberry, E. F. & Prockop, D. J. (1983*b*) Biochemistry **22**, 5213–5223
Bird, A. P. (1986) Nature (London) **321**, 209–213
Boedtker, H., Finer, M. & Aho, S. (1985) Ann. N.Y. Acad. Sci. **460**, 85–116
Brown, W. R. A. & Bird, A. P. (1986) Nature (London) **322**, 477–481
Byers, P. H. & Bonadio, J. F. (1985) in Genetic and Metabolic Diseases in Pediatrics (Lloyd, J. & Scriver, C. R., eds.), pp. 56–90, Butterworths, London
Cheah, K. S. E. (1985) Biochem. J. **229**, 287–303
de Wet, W. J., Pihlajaniemi, T., Myers, J., Kelly, T. E. & Prockop, D. J. (1983) J. Biol. Chem. **258**, 7721–7728
de Wet, W., Sippola, M., Tromp, G., Prockop, D., Chu, M.-L. & Ramirez, F. (1986) J. Biol. Chem. **261**, 3857–3862
Dickson, L. A., de Wet, W., Di Liberto, M., Weil, D. & Ramirez, F. (1985) Nucleic Acids Res. **13**, 3427–3438
Dixit, S. N., Seyer, J. M., Kang, A. H. & Gross, J. (1978) Biochemistry **17**, 5719–5722
Fietzek, P. P., Wendt, P., Kell, I. & Kuhn, K. (1972) FEBS Lett. **26**, 74–76
Galloway. D. (1982) in Collagen in Health and Disease (Weiss, J. B. & Jayson, M. I. V., eds.), pp. 528–557, Churchill–Livingstone, Edinburgh
Grobler-Rabie, A. F., Wallis, G., Brebner, D. K., Beighton, P., Bester, A. J. & Mathew, C. G. (1985) EMBO J. **4**, 1745–1748
Hayashi, T., Curran-Patel, S. & Prockop, D. J. (1979) Biochemistry **18**, 4182–4187
Hofmann, H., Fietzek, P. P. & Kuhn, K. (1978) J. Mol. Biol. **125**, 137–165
Kang, A. H., Bornstein, P. & Piez, K. A. (1967) Biochemistry **6**, 788–795
Messing, J. (1983) Methods Enzymol. **101**, 20–78
Mount, S. M. (1982) Nucleic Acids Res. **10**, 459–472
Myers, J. C., Dickson, L. A., de Wet, W., Bernard, M. P., Chu, M.-L., Di Liberto, M., Pepe, G., Sangiorgi, F. O. & Ramirez, F. (1983) J. Biol. Chem. **258**, 10128–10135
Ohkubo, H., Vogeli, G., Mudryj, M., Avvedimento, V. E., Sullivan, M., Pastan, I. & de Crombrugghe, B. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 7059–7063
Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. & Sharp, P. A. (1986) Annu. Rev. Biochem. **55**, 1119–1150
Peltonen, L., Palotie, A., Hayashi, T. & Prockop, D. J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 162–166
Piez, K. A. (1976) in Biochemistry of Collagen (Ramachandran, G. N. & Reddi, A. H., eds.), pp. 1–44, Plenum Press, New York
Prockop, D. J. & Kivirikko, K. I. (1984) N. Engl. J. Med. **311**, 376–386
Prockop, D. J. & Kuivaniemi, H. (1986) Rheumatology **10**, 246–271
Reed, R. & Maniatis, T. (1985) Cell **41**, 95–105
Ruskin, B., Krainer, A. R., Maniatis, T. & Green, M. R. (1984) Cell **38**, 317–331
Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Sippola, M., Kaffe, S. & Prockop, D. J. (1984) J. Biol. Chem. **259**, 14094–14100

Sykes, B., Wordsworth, P., Ogilvie, D., Anderson, J. & Jones, N. (1986) Lancet **ii**, 69–72

Tabor, S. & Richardson, C. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4767–4771

Tsipouras, P., Myers, J. C., Ramirez, F. & Prockop, D. J. (1983) J. Clin. Invest. **72**, 1262–1267

Vogeli, G., Ohkubo, H., Avvedimento, V. E., Sullivan, M., Yamada, Y., Mudryj, M., Pastan, I. & de Crombrugghe, B. (1980) Cold Spring Harbor Symp. Quant. Biol. **45**, 777–783

Yamada, Y., Avvedimento, V. E., Mudryj, M., Ohkubo, H., Vogeli, G., Irani, M., Pastan, I. & de Crombrugghe, B. (1980) Cell **22**, 887–892

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