Isolation and partial characterization of the entire human pro α 1(II) collagen gene

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ABSTRACT

Using a cDNA probe specific for the bovine Type II procollagen, a series of overlapping genomic clones containing 45 kb of contiguous human DNA have been isolated. Sequencing of a 54 bp exon, number 29, provided direct evidence that the recombinant clones bear human Type II collagen sequences. Localization of the 5' and 3' ends of the gene indicated that the human Type II collagen gene is 30 kb in size. This value is significantly higher than that of the homologous avian gene. The segregation of a polymorphic restriction site in informative families conclusively demonstrated that the Type II gene is found in a single copy in the human haploid genome. Finally, sequencing of a triple helical domain exon has confirmed that a rearrangement leading to the fusion of two exons occurred in the pro α 1(I) gene, following the divergence of the fibrillar collagens.

INTRODUCTION

Type II collagen, a genetically distinct member of the fibrillar collagens (Types I, II and III) is the major structural component of hyaline cartilage (1). This protein is composed of three identical α 1(II) chains and it shares with the other fibrillar collagens a similar molecular structure and biosynthetic pathway. The nascent chains of all three collagen types are synthesized as precursor molecules, procollagens, with a long α chain domain and two short terminal extensions which are removed extracellularly after secretion of the assembled triple helix (1). The central α -chain domains, approximately one thousand residues long, are composed of a repetitive tripeptide structure (Gly-X-Y)_{340 $\pm$ 2} (1).

The temporal expression of the Type II and Type I collagen genes is believed to play a fundamental role in the normal process of cartilage differentiation and bone formation (2). Defects at different levels of the ossification process have been postulated for the chondrodysplasias (3), a highly heterogeneous group of disorders. A structural defect in Type II collagen has been reported in at least one case of diastrophic dysplasia (4). Other studies have suggested that biosynthetic changes in Type II collagen are related

to the degeneration of the articular cartilage in the affected joints of osteoarthritic individuals (5). However, because of the complexity and sequential nature of the ossification process, very little is known about the pathogenesis of these acquired and inherited disorders.

In order to investigate the molecular basis of Type II collagen gene expression in normal and diseased states, several investigators have isolated cDNA and genomic clones coding for the pro α 1(II) collagen chain (6-13). The most extensive studies have been carried out on the chicken gene because of the relative ease of obtaining embryonic material in sufficient amounts for molecular cloning (6-11). More recently, however, some information has been obtained for portions of the pro α 1(II) gene from different mammalian species (12-13). In particular, we have isolated a cDNA clone (Bc-7) coding for the calf pro α 1(II) collagen chain and in turn used it for the identification of two bovine genomic clones covering the 3' end of the gene (Sangiorgi, F. et al., manuscript submitted). Here we report the use of Bc-7 for the isolation of a series of overlapping genomic clones encompassing the entire human pro α 1(II) collagen gene. Sequencing of selected areas has established that the gene is 30 kb long. Familial segregation of a restriction enzyme length polymorphism (RFLP) has demonstrated that the pro α 1(II) gene is present in a single copy in the human haploid genome. Finally, comparison of our data with those reported for a gene previously characterized as bearing "a1-like" collagen sequences (14) has revealed that the latter clone is indeed the human Type II procollagen gene.

MATERIALS AND METHODS

Gene Isolation

The probe used for the initial screening of the human library was Bc-7, a recombinant molecule containing 1011 bp of bovine pro α 1(II) collagen sequences (Sangiorgi, F. et al., manuscript submitted). The phage libraries utilized in these studies were both constructed in Charon 4A; one contained 15-20 kb partially digested Eco RI human genomic fragments (kindly provided by Dr. A. Bank, Columbia University), whereas the other was obtained by insertion of Alu I/Hae III partially digested DNA (kindly provided by Dr. T. Maniatis, Harvard University). Screening and isolation of the DNA from the positive phage clones was performed as described (15). Chromosomal walking was achieved by screening of the libraries with the appropriate genomic fragments subcloned in pBR322.

Southern and Northern blotting hybridizations

Transfer of nucleic acids onto nitrocellulose paper was done as previously described (15). Cross-hybridizations of the bovine probe to the human DNA were carried out at 40°C in 2x SSC (1x SSC:0.15M NaCl, 0.015 Sodium Citrate pH 6.8) 1x Denhart's solution, 100 µg/ml of sheared salmon sperm DNA and 50% formamide. Washing of the filters was done by sequentially decreasing the salt concentration from 2x SSC to 0.1x SSC and by increasing the temperature from 20°C to 60°C. For the cross-hybridization of the rat cDNA probe specific for the N-prepropeptide region of the pro α 1(II) gene, the salt concentration was increased to 4x SSC, whereas the temperature of the incubation was decreased to 37°C. In this case, washing of the filters was terminated at 0.2x SSC at 55°C. Hybridization and washing conditions for the other experiments were as previously detailed (15). In vitro labelling of the DNA probes was obtained by nick translation (16). Following mild alkaline hydrolysis (17) the RNA was labelled with [γ -³²P] ATP in the presence of T4 Kinase.

Primer extension

The synthetic oligonucleotide used in the primer extension experiments was derived from the heptapeptide Leu-Leu-Thr-Leu-Leu-Ile-Ala located between amino acid residues 12 and 18 in the rat pro α 1(II) N-propeptide (12). The oligonucleotide was used to prime cDNA synthesis using poly(A⁺) RNA isolated from a rat chondrosarcoma generously provided by Dr. V. Hascall (National Institutes of Health). The reaction was carried out in the presence of [α -³²P] labelled deoxynucleotides according to a modification of the procedures of Agarwal et al. (18) and Tate et al. (19). The product of the reaction was approximately 200 nucleotides long, as determined on a denaturing polyacrylamide gel, and it exhibited a specific activity of 0.8×10^7 cpm/µg.

RNA purification

Total poly(A⁺) RNA was extracted from a rat chondrosarcoma using the guanidinium HCl procedure (20), from frozen fetal bovine articular cartilage using a modification of the previously described method (21) and from cultured fibroblasts according to the published procedure (15).

DNA sequencing

The chemical modification method of Maxam and Gilbert (22) was used for the sequencing of the genomic clones. Sequencing of both strands was performed for most of the sequences presented in this paper.

RESULTS

Gene isolation and mapping

For the initial screening of the human genomic libraries the cDNA clone

Fig. 1. Southern blotting analysis of human genomic DNA digested with Eco RI and hybridized to the entire bovine probe Bc-7 (lane 1), to the 3' segment of Bc-7 (lane 2) and to the 5' segment of Bc-7 (lane 3).

Bc-7 was used. This recombinant molecule contains a eucaryotic insert of 1 kb, coding for almost 75% of the C-propeptide domain of the bovine Type II collagen. Interspecies comparison has shown that the sequences of Bc-7 exhibit an 85% homology with those determined for the homologous avian chain (Sangiorgi, F. et al., manuscript submitted). Prior to the screening of the genomic libraries we optimized the cross-hybridization conditions of the bovine probe to the corresponding human sequences by Southern blotting analysis of Eco RI digested total human nuclear DNA. These experiments showed a unique pattern even under stringent conditions of hybridization and washing (Fig. 1). More precisely, by this analysis two bands, 3.7 kb and 4.0 kb in size, were identified in the human genome. It should be noted that in Bc-7 a unique Eco RI site, 35 bp from the termination codon, generates two subfragments of almost equal length, one specific for the 5' coding region and the other for the 3' untranslated region. Hence, we used these two subfragments as probes to assess whether the human gene possessed the same Eco RI site and to orient the two genomic bands. As is shown in Figure 1, the 5' subfragment of Bc-7 hybridized with the 3.7 kb Eco RI band, whereas the 4.0 kb was recognized by the 3' subfragment of Bc-7. Based on these results we proceeded to screen the Eco RI genomic library which yielded two positive clones, Pis 8 and Pis 36. Eco RI digestion of the DNA from Pis 8 generated four fragments: 4.5 kb, 4.0 kb, 3.7 kb and 2.5 kb. The DNA of Pis 36 showed a slightly different Eco RI pattern: 4.5 kb, 3.8 kb, 3.7 kb and 2.5 kb. Con-

Fig. 2. Northern blotting analysis of poly(A⁺) RNA isolated from cartilaginous and non-cartilaginous tissues and hybridized to Bc-7 (lanes 1 and 2), the 3.7 kb Eco RI subclone of Pis 8 (lane 3), the 4.0 kb Eco RI subclone of Pis 8 (lane 4) and the 3.8 kb Eco RI subclone of Pis 36 (lane 5). Fibroblast RNA is in lane 2, whereas bovine fetal cartilage RNA is in the other four lanes. The size of the mRNA is expressed in nucleotides.

sistent with the previous Southern blotting analysis of total genomic DNA, the 5' end segment of Bc-7 hybridized to the 3.7 kb fragments of Pis 8 and Pis 36. On the other hand, the 3' segment of Bc-7 recognized both the 4.0 kb fragment of Pis 8 and the 3.8 kb fragment of Pis 36.

Several possibilities existed to explain the difference observed between the Eco RI patterns of Pis 8 and Pis 36. First, one could have argued for the existence of a polymorphic Eco RI site in Pis 36, which shortened the length of the 4.0 kb fragment to 3.8 kb. We excluded this possibility because Pis 36 did not contain an additional 0.2 kb Eco RI fragment and because after extensive restriction mapping the 4.5 kb and 2.5 kb Eco RI fragments were placed downstream to the 4.0 kb (Fig. 3).

A second alternative was the existence of two distinct genes with slightly different Eco RI patterns, a notion, however, not substantiated by our previous Southern blotting analysis of total genomic DNA. In addition, Northern blotting hybridizations of the 4.0 kb and 3.8 kb as well as the 3.7 kb fragments to RNA extracted from bovine articular cartilage, exhibited a pattern identical to that obtained with Bc-7 (Fig. 2). This result demonstrated that the genomic fragments behaved in a manner consistent with the size, tissue specificity and quantitative representation predicted for a pro α 1(II) collagen species. Finally, partial sequencing of the 4.0 kb and 3.8 kb DNA from the two Eco RI sites showed complete identity between the two fragments.

Fig. 3. Restriction map of the 45 kb of chromosomal DNA contained within the overlapping genomic clones. The sites shown refer to the following enzymes: B: Bam HI; C: Cla I; E: Eco RI; H: Hind III; K: Kpn I; S: Sph I; X: Xba I; Xh: Xho I. The numbers beneath refer to the size of the Eco RI fragments expressed in kilobases. The asterisk identifies the Eco RI site common to both the genomic clones and the cDNA Bc-7, whose position relative to the genomic clones is signified by the open bar below the restriction map. Similarly, the open triangle locates the 200 bp deletion of Pis 36, whereas the black bars indicate the presence of repetitive sequences. The dotted line above the restriction map shows the location of the Bam HI fragment used in the RFLP experiments. The relative position of the Type II gene with respect to the 45 kb of chromosomal DNA is indicated by the double arrow on top of the map. At the bottom of the map are the relative positions of the genomic clones and the scale expressed in kilobases.

A third possibility was that the Pis 8 and Pis 36 were derived from two alleles with a short polymorphic 200 bp DNA deletion. We proved that this was indeed the case by extensive restriction mapping of the 4.0 kb and 3.8 kb fragments in conjunction with Southern blotting analysis of the DNA from several individuals. These experiments located the deletion between an Xho I and an Xba I site of Pis 8 (Fig. 3). The nature of the deletion and its frequency in the population will be discussed elsewhere (Tsipouras, P. et al., in preparation). Direct confirmation of the identity of the genomic clones with Bc-7 was obtained by selective sequencing of the 3.7 kb and 4.0 kb Eco RI fragments of Pis 8 (Figs. 4A, 5).

The 3.7 kb Eco RI subclone of Pis 8 was used for a second genomic screening which resulted in the isolation of Pis 2 from the Alu I/Hae III

Fig. 4. Detailed restriction maps of the regions comprising the Eco RI fragments (A,B,C,D) whose areas have been selectively sequenced (see Figs. 3,6, and 8). The letters indicate the following restriction sites: B: Bam HI; Bg: Bgl II; E: Eco RI; H: Hind III; K: Kpn I; P: Pst I; Pv: Pvu II; S: Sph I; Sc: Sac I; SII: Sac II; Sm: Sma I; Xh: Xho I. The exons are identified by the black boxes and they are numbered underneath in a sequential order beginning from the 3' end of the gene. The sizes of four triple helical exons are indicated above them. The cross hatched box of exon 1 signifies the non-coding sequences, whereas in exons 30 and 52 it indicates that they have been partially sequenced.

library. The DNA of Pis 2 was subjected to extensive restriction mapping in parallel with hybridization of its different genomic fragments to Southern blotted human DNA cleaved with various restriction enzymes. From this analysis a highly polymorphic Hind III site was identified (Tsipouras, P. et al., in preparation). This finding allowed us to demonstrate the single copy nature of the human pro α (II) gene by analyzing the segregation of the Hind III polymorphism in informative families. In these experiments, we hybridized total human DNA digested with Hind III to the 2 kb Bam HI:Bam HI subclone of Pis 2, which overlaps the 9.2 kb and 3.7 kb Eco RI fragments (Fig. 3). When the Hind III site was absent in the nuclear DNA as in Pis 2, the probe hybridized to a unique band approximately 13 kb in size. On the other hand, the presence of the Hind III site resulted in two comigrating fragments, 6.7 kb in length (Fig. 6).

At this point we came to the realization that the restriction map of Pis 2 closely resembled that of an "a1-like" collagen gene previously isolated by Weiss et al. from a human cosmid library using a chicken pro α (I) cDNA probe (14). In their report, the authors excluded the possibility that this cosmid clone was the pro α (II) collagen gene based on the sequencing of a selected

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1000                                1014 1c
▽GCT CCT CCT GGA AAT C C T T C C T C C C C C A C
Gly Pro Pro Gly Asn Pro Gly Pro Pro Gly Pro Pro Gly Pro Gly Ile Asp Met Ser
Thr

A C G T A C G
GCT TTT GCT GGC TTA GGC CCG AGA GAG AAG GGC CCC GAC CCC CTG CAG TAC ATG CCG GGC GAC CAG
Ala Phe Ala Gly Leu Gly Pro Arg Glu Lys Gly Pro Asp Pro Leu Gln Tyr Met Arg Ala Asp Gln
Gln Thr

30c
GCG GGC GGT GGC CTG AGA CAG CAT GAC GGC GAG GTG GAT GCC ACA CTC AAG TCC CTC AAC AAC CAG
Ala Ala Gly Gly Leu Arg Gln His Asp Val Ala Glu Val Asp Ala Thr Leu Lys Ser Leu Asn Asn Gln

60c
ATT GAG AGC ATC CCG AGC CCC GAG GGC TCC CCG AAG AAC CCT C A G C C C A C
Ile Glu Ser Ile Arg Ser Pro Glu Gly Ser Arg Lys Asn Pro Ala Arg Thr Cys Arg Asp Leu Lys
Lys

90c
CTC TCC CAC CCT GAG TGG AAG AGT GGA TAC TGG ATT GAC GGC AAC CAA GGC TCC ACC TTG GAC
Leu Cys His Pro Glu Trp Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp

C A A A
GCG ATG AAG GTT TTC TGC AAC ATG GAG ACT GGC GAG ACT TGC GTC TAC CCG AAT CCA GCA AAC GTT
Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys Val Tyr Pro Asn Pro Ala Asn Val
Ile Thr Ser Ser Ile

120c
G
CGC AAG AAG AAC TGG TGG AGC AGC AAG AGC AAG GAG AAG AAA CAC ATC TGG TTT GGA GAA ACC ATC
Pro Lys Lys Asn Trp Trp Ser Ser Lys Ser Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Ile
Arg Thr

C C T C
AAT GGT GGG TTC CAT
Asn Gly Gly Phe His

150c
C C C
▽TTC AGC TAT GGA GAT GAC AAC CTG GCT CCC AAC ACC GGC GAC GTC CAG ATG ACC TTC CTG CCG CTG
Phe Ser Tyr Gly Asp Asp Asn Leu Ala Pro Asn Thr Ala Asp Val Gln Met Thr Phe Leu Arg Leu
Glu Ser Ile

180c
CTG TCC GGC GAG GGC TCC CAG AAC ATC ACC TAC CAC TGC AAG AAC AGC ATT GGC TAC ATG GAC GAG
Leu Ser Ala Glu Gly Ser Gln Asn Ile Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Met Asp Glu
Thr Val

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A A G G A A C
GCG GCT GGC AAC CTC AAG AAG GGC CTG CTC ATC CAG GGC TCC AAT GAC GTG GAG ATC CCG GCA GAG
Ala Ala Gly Asn Leu Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Ile Arg Ala Glu
Glu Thr

C C G C T T G C
GGC AAT AGC AGG TTC ACG TAC ACT GGC CTG AAG GAT GGC TGC ACG AAA CAT ACC GGT AAG TGG GGC
Gly Asn Ser Arg Phe Thr Thr Ala Leu Lys Asp Gly Cys Thr Lys His Thr Gly Lys Trp Gly
Ser Val Glu

240c
G G G
AAG ACT GTT ATC GAG TAC CCG TCA CAG AAG ACC TCA CCG CTC CCC ATC ATT GAC ATT GCA CCC ATG
Lys Thr Val Ile Glu Tyr Arg Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met
Val

270c
T C A G T G T C T T C A
GAC ATA GGA GGC CCG GAG CAG GAA TTC GGT GTG GAC ATA GGG CCT GTC TCC TTC TTG TAA
Asp Ile Gly Gly Pro Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys Phe Leu ***
Ala Asp

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Fig. 5. DNA and amino acid sequences of the four exons coding for the C-propeptide domain of the proal(II) chain. Only the differences with the avian gene are shown. Underlined are the cysteinyl residues and the carbohydrate attachment site (dotted line). The triangles demarcate the exons, the vertical bar separates the triple helical domain from the C-terminal telopeptide. The arrow identifies the C-proteinase cleavage site. The three asterisks emphasize the termination codon for translation.

Fig. 6. Southern blotting analysis of the segregation of the Hind III RFLP in an informative family. Lane 1: offspring heterozygous for the presence of the site; lane 2: parent homozygous for the absence of the site, and lane 3: parent homozygous for the presence of the site. The approximate sizes of the hybridizing bands were estimated by comparison with λ DNA digested with Hind III and run in a parallel lane.

area. To demonstrate identity between our clone and the "α1-like" gene we sequenced the same chromosomal region and found complete homology of two exons (numbers 8 and 9) as well as of the intervening sequences (Figs. 4A, B and 7).

Previously, we identified Bc-7 as a Type II collagen specific clone because of its high degree of sequence homology with the C-propeptide domain of the avian gene (Sangiorgi, F. et al., manuscript submitted). Similarly, Strom et al. used this type of evidence to prove that their genomic clone coded for the 3' end of the human proα1(II) gene (13). Although highly suggestive, neither of the investigations provided the nucleotide sequences for those regions of the α1(II) chain whose amino acid sequences have been determined (23,24). Analysis of the 5' foremost section of Pis 2 identified a 54 bp exon, number 29, coding for amino acid residues 361 to 378 (Figs. 4B and 7). The amino acid translation of exon 29 was compared to the published amino acid sequences of the bovine α1(II) chain and found to be identical (24). Moreover, sequencing 5' from exon 29 identified a 60 bp open reading frame, exon 30, whose sequences exhibited a high level of homology with the corresponding segment of the proα1(I) gene. This observation was well in agreement with evidence suggesting that the proα1(I) and proα1(II) collagens are more homologous than are the proα1(I), proα2(I) and proα1(III) collagens

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      856
tgacag GGT AAT CGT GGT GAA ACC GGT GCT GTG GGA GCT CCT GGA ACC CCT GGG CCC CCT GGC TCC CCT
      Gly Asn Arg Gly Glu Thr Gly Ala Val Gly Ala Pro Gly Thr Pro Gly Pro Pro Gly Ser Pro

      GGC CCC GCT GGT CCA ACT GGC AAG CAA GGA GAC AGA GGA GAA GCT gtaagtatctctggaattcactaaagcc
      Gly Pro Ala Gly Pro Thr Gly Lys Gln Gly Asp Arg Gly Glu Ala

      gcttccctcgcgcggtggggctgaggcagttcctgggttttccagtgctctggactaaggagcagtggtgcccagatgcagaggagg

      ccccacctgtcctgcttttctctagcctgcgtcactctctctcag      892 GGT GCA CAA GGC CCC ATG GGA CCC TCA GGA
      Gly Ala Gln Gly Pro Met Gly Pro Ser Gly

      909
CCA GCT GGA GGC GGG GGA ATC CAG gtgagta
Pro Ala Gly Ala Arg Gly Ile Gln

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      586
ag GGC CTG ACA GGT CCC ATT GGC CCC CCT GGC CCA GGC GGT GCT AAC GGC GAG AAG gtgagt
      Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala Gly Ala Asn Gly Glu Lys

      603

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      361      *      *      378
ag GGT CTC ACT GGC GGC CCT GGT GAT GCT GGT CTT CAA GGC AAA GTT GGT CCC TCC gtaagt
      Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly Lys Val Gly Pro Ser

      341
... CCC AAG GGA GGC AAC GGT GAC CCT GGC CGT CCT GGA GAA CCT GGC CTT CCC GGA GGC CGG gtaagt
      ... Pro Lys Gly Ala Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro Gly Ala Arg

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Fig. 7. DNA and amino acid sequences of five triple helical exons. The amino acid residues are numbered above and some of the intervening sequences are shown (small caps). The asterisks signify possible ambiguities.

(25). Thus, these data conclusively proved that the gene coded for the $\alpha 1$ chain of human Type II procollagen.

The same 5' subclone of Pis 2 was used for a further genomic screening which led to the isolation of a clone from the Alu I/Hae III library, Pis 10, and a clone from the Eco RI library, Pis 14. The restriction map of the two recombinants and their relationship to Pis 2 is shown in Fig. 3. Comparison with the map of the " $\alpha 1$ -like" gene (14) revealed one major difference. Contrary to the placement by Weiss et al. of the 0.8 kb Eco RI fragment immediately 5' to the 9.2 kb fragment, our restriction mapping suggested that this small Eco RI fragment was 5' to the 7.3 kb Eco RI fragment. In order to resolve this discrepancy, the 0.8 kb Eco RI subclone of Pis 10 was used for the screening of the Alu I/Hae III library which led to the isolation of Pis 93. Southern blotting hybridizations showed that Pis 93 contained the same 0.8 kb fragment of Pis 10, but it did not overlap with the 9.2 kb Eco RI fragment. Thus, this analysis amended the incorrect location of the 0.8 kb fragment as it appeared in the restriction map of the " $\alpha 1$ -like" gene (14). Finally, the relative locations of three repetitive sequences within the 45 kb of the overlapping clones were identified by hybridization to nick translated total DNA (Fig. 3).

Fig. 8. Identification of exon 52. Left, ethidium bromide staining of the 4.8 kb Eco RI subclone cleaved with Pst I and Kpn I (see also Fig. 3C) and right, autoradiography of the same DNA after Southern blotting hybridization to the primer-extended rat cDNA probe.

Determination of the 5' end of the gene

In order to locate the 5' end of the gene we first hybridized the Eco RI digests of the DNA from Pis 10, Pis 14 and Pis 93 to *in vitro* labelled total poly(A⁺) RNA purified from bovine fetal articular cartilage. While the 7.3 kb, 5.2 kb and 4.8 kb fragments gave a positive signal, the 2.0 kb and 0.8 kb fragments were negative (data not shown). Based on the mapping of the three genomic clones we concluded that the 0.8 kb was negative because it contained intervening sequences. On the other hand, the lack of hybridization of the 2.0 kb Eco RI fragment could have been explained by the presence of either intervening or 5' flanking sequences. To discriminate between these two alternatives and to establish the possible location of the 5' most segment of the gene within the 4.8 kb Eco RI subclone, we used a single stranded cDNA coding for the last 200 nucleotides of the rat pro α 1(II) collagen (12). The rationale for this approach was based on the high sequence homology of the human and bovine pro α 1(II) collagens as well as the human and the avian genes. Thus, we constructed an artificial 21-mer coding for the heptapeptide which Kohno et al. have found to be located between amino acid residues 12 to 18 in the last exon of the rat pro α 1(II) gene (12). Hybridization of the rat probe to the 4.8 kb subclone cleaved with different restriction enzymes suggested

Fig. 9. DNA and nucleotide sequences of the three N-prepropeptide exons. Shown are only the differences with the corresponding region of the rat gene. The vestigial exon is underlined by the dotted line. The asterisks signify possible ambiguities.

that some sequence similarities were present within the 1.3 kb Pst I:Kpn I fragment of the 4.8 kb subclone (Figs. 4C and 8). Sequencing of this area identified a 67 bp open-reading frame potentially coding for 22 1/3 amino acid residues. Comparison with the rat sequences showed that the human peptide corresponded to amino acid residues 7 to 28 of the rodent N-prepropeptide domain (Figs. 4C and 9). This notion was further supported by the high level of nucleotide (90%) and amino acid (82%) homology observed between the two species in this region of the gene. Based on the remarkable conservation of the exon arrangement of the fibrillar collagen genes, we believe that this exon represents the last coding unit of the pro α 1(II) gene, for which we tentatively assigned the number 52. From these data we therefore concluded that the 45 kb of contiguous DNA of the overlapping phage clones contained the entire human Type II procollagen gene, which is approximately 30 kb long.

Additional information about the arrangement of the exons coding for the N-propeptide domain was gathered from sequencing around the unique KpnI site of the 7.3 kb Eco RI fragment common to Pis 10 and Pis 93 (Figs. 4D and 9). Two small open-reading frames were found at approximately 120 bp 5' to the Kpn I site and 84 bp 3' to the Kpn I site (Figs. 4D and 9). These two exons, 17 bp and 33 bp respectively, code for a segment corresponding to amino acid

TABLE I
Exon-intron arrangement of the C-propeptide
region of the human fibrillar collagen genes

	<u>Exon 1</u>	<u>Intron 1</u>	<u>Exon 2</u>	<u>Intron 2</u>	<u>Exon 3</u>	<u>Intron 3</u>	<u>Exon 4</u>	<u>Exon/Intron Ratio</u>	
								<u>Human</u>	<u>Chicken</u>
Pro α 1(I)	144	170	243	280	191	210	238	1.5	N.D.
Pro α 2(I)	144	770	243	900	185	500	214	0.36	0.34
Pro α 1(II)	144	500	243	350	188	600	244	0.56	0.73
Pro α 1(III)	144	600	243	300	188	1000	235	0.42	0.45

The sizes of exon 1 refer only to the coding elements. The sizes of exon 4 do not include the C-terminal portion of the triple helical domain. The sizes of most of the introns have been approximated on the basis of R-looping analysis, partial DNA sequencing and restriction mapping of the genomic fragments. The data presented in the table were extracted from several investigations (8,10,15,28,31,24,42).

residues 29 to 45 of the rat N-propeptide. Comparison between the human and rodent gene showed only 7 nucleotide changes resulting in two amino acid replacements. Thus, the interspecies homology for these two exons is identical to that previously discussed for exon 52. In line with those assumptions, the two exons were identified as numbers 50 and 51.

Interestingly, 20 bp upstream from exon 50 we found another open-reading frame, 29 bp long, coding for 3 Gly-X-Y repeats and ending with a split codon (Fig. 9). Intronic sequences coding for collagenous elements have also been identified in the mouse and human pro α 1(I) collagen genes (26, Ramirez, F., unpublished data). Monson et al. have reasoned that these unusual elements may represent remnants of the complex evolutionary history of the collagen genes (26). Unlike those found in the pro α 1(I), the vestige of the Type II gene is flanked by accepted variations of the intron splice sequences (27,28). However, although the pro α 1(II) vestige has the potential to be processed into the mature mRNA, its amino acid sequence translation does not correspond to any segment of the rat N-prepropeptide (12). Furthermore, the translation of this element would put the reading frames of both exon 51 and 50 out of phase. We presently do not have any conclusive explanation for this unusual finding, although one could argue for the lack of other DNA elements which may be necessary for proper exon processing.

DISCUSSION

Pro α 1(II) C-propeptide

As originally noted by Wozney et al. for the chicken pro α 2(I) (29), the C-propeptide domain of all fibrillar procollagen genes is divided into four

TABLE II
Codon usage for the human fibrillar collagen α -chains

Third Base	Gly				Pro				Ala			
	$\alpha 1(I)$ (261)	$\alpha 1(II)$ (38)	$\alpha 2(I)$ (166)	$\alpha 1(III)$ (58)	$\alpha 1(I)$ (179)	$\alpha 1(II)$ (32)	$\alpha 2(I)$ (92)	$\alpha 1(III)$ (35)	$\alpha 1(I)$ (32)	$\alpha 1(II)$ (12)	$\alpha 2(I)$ (55)	$\alpha 1(III)$ (12)
U	50%	37%	56%	47%	57%	53%	66%	65%	74%	58%	84%	58%
C	30%	37%	20%	17%	40%	38%	21%	14%	21%	33%	3%	16%
A	18%	24%	13%	31%	3%	9%	10%	25%	5%	9%	7%	25%
G	2%	2%	4%	5%	0%	0%	0%	0%	0%	0%	0%	0%

The values in parentheses indicate the number of codons examined.

exons coding distinct functional or conformational segments of this region of the protein molecule (28). This notion is supported by several lines of experimental evidence suggesting that the C-propeptides play a key role in the selection and correct alignment of the pro α chains to form the procollagen trimers (30). In Table I is shown a summary of our data relating to the exon-intron arrangement of the region coding for the C-propeptide domains of the human fibrillar procollagens. Regardless of the size differences of exons 3 and 4, the most striking dissimilarities are seen in the relative exon/intron ratios of the four genes (Table I). Comparison between the human and avian genes revealed almost identical exon/intron ratios in the pro $\alpha 2(I)$ and pro $\alpha 1(III)$, but not in the pro $\alpha 1(II)$ genes. In this context, it should be noted that Strom et al. (13) have reported that in the human Type II collagen gene the distance between a triple helical exon coding for amino acid residues 694 to 711 and the 3' end of exon 4 spans approximately 7.6 kb. This value differs from the 2.9 kb of the avian counterpart and the 4.0 kb we estimated by restriction mapping and DNA sequencing. We believe that this discrepancy is due to a mapping error on the part of these investigators.

Interspecies comparison between the human and the homologous sequences from the bovine and chicken genes indicated a high degree of evolutionary conservation, 96% and 88% respectively. An interesting amino acid change was observed at position 153 (Ser to Ala) in the mammalian C-propeptides when compared to the avian chain (8, Sangiorgi, F. et al., manuscript submitted). This change eliminates the formal possibility that a second glycosylation site (Asn-Leu-Ser) may be present in the Type II C-propeptide. Sequencing of 158 bp of the 3' untranslated region indicated an almost complete identity with

the homologous segment of the bovine gene. Based on this finding and on the mRNA sizing by Northern blotting hybridization (Fig. 2), we extrapolated that the total length of exon 1 should not significantly differ from the 580 bp we reported for the calf gene. Interestingly, the pro α 1(II) gene is the only one among the fibrillar collagens which does not exhibit polymorphic mRNA transcripts (31-34).

Triple helical domain exons

Although the aim of this study was to identify the exact nature of the overlapping genomic clones, sequencing of selected areas of the region coding for the triple helical domain led to an interesting structural finding related to the evolution of the fibrillar collagen genes. The molecular analysis of this subfamily of genes has revealed that they share a remarkably similar arrangement of the numerous exons coding for the triple helical domain (28). Thus far, only one major difference has been identified: the presence of a single 108 bp exon in the pro α 1(I) gene coding for amino acid residues 568 to 603 (exon 19) which, in the pro α 2(I) and pro α 1(III) genes, is encoded by two 54 bp exons (15,26,28,35 and de Wet et al. in preparation). We have now determined that in the human pro α 1(II) gene the size of exon 19 is also 54 bp. Thus, we concluded that the exon fusion observed in the pro α 1(I) gene occurred sometime after the duplication and divergence of the four fibrillar collagen genes from the ancestral multiexon unit. In this context we should add that a similar recombinational event has also occurred in the triple helical segment of exon 4 of the pro α 1(III) gene. In fact, in the human gene and unlike in any of the other collagens, including the chicken pro α 1(III) gene, this portion of exon 4 has an extra Gly-X-Y triplet resulting in an unusual coding segment 63 bp long (35).

In the various collagens there is a constant pattern, maintained throughout evolution, for the third position preference of the proline, alanine and glycine codons of the triple helical domain exons (28). Thus, we analyzed this feature in the available sequences of the α 1(II) chain and compared it to those previously determined for the human α 1(I), α 2(I) and α 1(III) chains, (Table II) (35). Although the number of α 1(II) codons examined was relatively small, it is clear that unlike the pro α 1(III) gene, the pro α 1(II) does not significantly differ from the pro α 1(I) and pro α 2(I) collagens for the first and second choice in the wobble position.

N-terminal domain

The N-terminal portion of the fibrillar collagens has been divided into four distinct subdomains: the signal peptide, an N-terminal globular region,

a triple helical segment and a short non-helical part (1). Variations in the size and composition of the N-terminal domains of the collagen molecules have been reported for the different chains as well as for the same chain in different species (1). For example, in the globular domain of the human, mouse and chicken pro α 1(I) N-terminal region [pN α 1(I)], 12, 9 and 7 acidic amino acid residues are present, respectively (15). Similar variations have been reported for the avian and bovine pro α 1(III) chains (36). In the pN α 2(I) the globular region is almost absent, resulting in a significantly shorter N-terminal propeptide (1). As in the pro α 2(I) chain, Type II collagen lacks the cluster of cysteinyl residues in the globular domain which in the pN α 1(I) and pN α 1(III) are believed to participate in intrachain disulfide bonds (1,12). Furthermore, the Gly-X-Y repeats of the collagenous region of the pN α 1(II), which is much larger than that of the other fibrillar collagen chains, are interrupted once between residues 44 and 47 (12). At the gene level it has been noted that, regardless of these structural differences, six exons are present in the region coding for the collagen N-prepropeptides (15,28). Moreover, the transitions between the four N-terminal subdomains and between the N-terminal propeptide and the signal peptide as well as the large triple helical domain of the collagen, are encoded by junction exons (15,28). Although preliminary, our data seem to suggest that a similar exon distribution is present in the Type II gene. Interestingly, the short non-collagenous segment which interrupts the Gly-X-Y repeats of the pN α 1(II) is split between two exons. Investigations are currently in progress aimed at defining in more detail the structure of this portion of the pro α 1(II) collagen gene.

Gene size and copy number

Although the pro α 1(I), pro α 2(I), pro α 1(II) and pro α 1(III) collagen genes have maintained a complex structural similarity during evolution, they have diverged from each other both at the level of their nucleotide sequences and in the length of their introns (15,28). It has been argued that, as in the α -like and β -like globin gene clusters (37), the size differences may be the result of the distinct chromosomal origins of the four genes (38, Huerre-Jeanpierre, C. et al., manuscript submitted). Comparative analysis of the pro α 1(I), pro α 2(I) and part of the pro α 1(III) collagens has shown that the sizes of these genes do not seem to vary significantly in different species (15,31,35). It was therefore surprising to find that the total length of the human pro α 1(II) gene is similar to that of the pro α 2(I) gene (28, de Wet, W. et al., in preparation), and greater than that reported for the avian counterpart (8). To the best of our knowledge, a similar phenomenon has been

observed only in the mammalian dihydrofolate reductase (DHFR) gene (39). The five introns of the DHFR gene are located in homologous positions and show a strong length divergence in man, mouse and Chinese hamster. However, and unlike the $\alpha 1(\text{II})$ collagen, the overall length of the DHFR gene is approximately the same (25-30 kb) in the three species (39).

The familial segregation of a highly polymorphic Hind III site has proved that the $\alpha 1(\text{II})$ gene, which is located on the segment 12q131-12q132 of chromosome 12 (Huerre-Jeanpierre, C. et al., manuscript submitted), is present in a single copy in the haploid human genome. This notion does not seem to substantiate the idea that the minor Type II collagen found in bovine nasal cartilage (40) is indeed a separate gene product, unless one postulates that its sequences significantly diverged from those of the major Type II collagen gene. Conceivably, the biochemical heterogeneity of the $\alpha 1(\text{II})$ chains (2,40,41) could also be explained by alternative splicing of a common mRNA precursor. A more detailed comparative analysis of the $\alpha 1(\text{II})$ gene structure in different vertebrates and in different tissues in the same species will provide some insights into this important question.

SUMMARY

In conclusion, we have isolated the entire human $\alpha 1(\text{II})$ collagen gene and found that it is 30 kb in length and present in a single copy in the human haploid genome. Sequencing of selected areas has determined the primary structure and the exon/intron arrangement of the regions coding for the entire C-propeptide domain and part of the N-prepropeptide domain. Pairwise and interspecies comparisons have confirmed some of the evolutionary features of the collagen multigene family. Experiments are currently in progress aimed at completing the characterization of the $\alpha 1(\text{II})$ collagen gene, including its 5' promoter region. These studies are an important prerequisite for elucidating the nature of those factors regulating Type II expression in physiological and pathological conditions.

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REFERENCES

1. Bornstein, P. and Sage, H. (1980) *Ann. Rev. Biochem.* 49, 957-1003.
2. Mayne, R. and von der Mark, K. (1983) in *Cartilage. Structure Function and Biochemistry*, Hall, B.K., Ed. Vol. 1. pp. 181-214, Academic Press, New York.
3. Horton, W.A. (1984) in *Practice of Pediatrics*, Kelley, V.C., Ed. Vol. 7. pp. 1-15, Harper and Row, Philadelphia.
4. Stanesco, R., Stanesco, V. and Maroteaux, P. (1982) *Collagen Rel. Res.* 2, 111-116.
5. Nimni, M. and Deshmukh, K. (1973) *Science* 181, 751-753.
6. Vuorio, E., Sandell, L., Kravis, D., Sheffield, V.C., Vuorio, T., Dorfman, A. and Upholt, W.B. (1982) *Nucleic Acids Res.* 10, 1175-1192.
7. Sandell, L.J., Yamada, Y., Dorfman, A. and Upholt, W.B. (1983) *J. Biol. Chem.* 258, 11617-11621.
8. Sandell, L.J., Prentice, H.L., Kravis, D. and Upholt, W.B. (1984) *J. Biol. Chem.* 259, 7826-7834.
9. Ninomiya, Y., Showalter, A.M., van der Rest, M., Seidah, N.G., Chretien, M. and Olsen, B.R. (1984) *Biochemistry* 23, 617-624.
10. Young, M.F., Vogeli, G., Nunez, A.M., Fernandez, M.P., Sullivan, M. and Sobel, M.E. (1984) *Nucleic Acids Res.* 12, 4207-4228.
11. Lukens, L.N., Frischauf, A.M., Pawlowski, P.J., Brierley, G.T. and Lehrach, H. (1983) *Nucleic Acids Res.* 11, 6021-6039.
12. Kohno, K., Martin, G.R. and Yamada, Y. (1984) *J. Biol. Chem.* 259, 13668-13673.
13. Strom, C.M. and Upholt, W.B. (1984) *Nucleic Acids Res.* 12, 1025-1038.
14. Weiss, E.H., Cheah, K.S.E., Grosveld, F.G., Dahl, H.H.M., Solomon, E. and Flavell, R.A. (1982) *Nucleic Acids Res.* 10, 1981-1994.
15. Chu, M.-L., de Wet, W., Bernard, M., Ding, J.F., Morabito, M., Myers, J., Williams, C. and Ramirez, F. (1984) *Nature* 310, 337-340.
16. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in *Molecular Cloning. A Laboratory Manual*. pp. 270-294, Cold Spring Harbor Laboratory, New York.
17. Maizels, N. (1976) *Cell* 9, 431-438.
18. Agarwal, K.L., Brunstedt, J. and Noyes, B.E. (1981) *J. Biol. Chem.* 256, 1023-1028.
19. Tate, V.E., Finer, M.H., Boedtker, H. and Doty, P. (1983) *Nucleic Acids Res.* 11, 91-103.
20. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* 18, 5294-5299.
21. Ramirez, F., Natta, C., O'Donnell, J.V., Canale, V., Bailey, G., Sanguen-sermsri, T., Maniatis, G.M., Marks, P.A. and Bank, A. (1975) *Proc. Natl. Acad. Sci. USA* 72, 1550-1554.
22. Maxam, A.M. and Gilbert, W. (1980) *Methods in Enzymol.* 65, 499-560.
23. Bornstein, P. and Traub, W. in *The Proteins*. Neurath, H. and Hill, R.L., Eds. Vol. 4 pp. 411-632, Academic Press, New York.
24. Butler, W.T., Finch, J.E. and Miller, E.J. (1977) *Biochemistry* 16, 4981-4990.
25. Fuller, F. and Boedtker, H. (1981) *Biochemistry* 20, 996-1006.
26. Monson, J.M., Friedman, J. and McCarthy, B.J. (1982) *Mol. Cell Biol.* 2, 1362-1371.

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27. Breathnach, R. and Chambon, P. (1981) *Ann. Rev. Biochem.* 50, 349-383.
 28. Tate, V., Finer, M., Boedtker, H. and Doty, P. (1982) *Cold Spring Harbor Symp. Quant. Biol.* 47, 1039-1049.
 29. Wozney, J., Hanahan, D., Tate, V., Boedtker, H. and Doty, P. (1981) *Nature* 294, 129-135.
 30. Dickson, L.A., Pihlajaniemi, T., Deak, S., Pope, F.M., Nicholls, A., Prockop, D.J. and Myers, J.C. (1984) *Proc. Natl. Acad. Sci. USA* 81, 4524-4528.
 31. Myers, J.C., Dickson, L.A., de Wet, W.J., Bernard, M.P., Chu, M.-L., Di Liberto, M., Pepe, G., Sangiorgi, F.O. and Ramirez, F. (1983) *J. Biol. Chem.* 258, 10128-10135.
 32. Chu, M.-L., de Wet, W., Bernard, M. and Ramirez, F. (1985) *J. Biol. Chem.*, in press.
 33. Aho, S., Tate, V. and Boedtker, H. (1983) *Nucleic Acids Res.* 11, 5443-5450.
 34. Merlino, G.T., McKeon, C., de Crombrughe, B. and Pastan, I. (1983) *J. Biol. Chem.* 258, 10041-10048.
 35. Chu, M.-L., Weil, D., de Wet, W., Bernard, M., Sippola, M. and Ramirez, F. (1985) *J. Biol. Chem.*, in press.
 36. Timpl, R. and Glanville, W.R. (1981) *Clin. Orthop. Relat. Res.* 158, 224-241.
 37. Smithies, O., Bleche, A.E., Shen, S., Slightom, J.L. and Vanin, E.L. (1981) in *Levels of Genetic Control in Development*, pp. 185-200, A. Liss Inc., New York.
 38. Huerre, C., Junien, C., Weil, D., Chu, M.-L., Morabito, M., Foubert, C., Myers, J.C., Van Cong, N., Gross, M.S., Prockop, D.J., Boue, A., Kaplan, J.L., de la Chapelle, A. and Ramirez, F. (1982) *Proc. Natl. Acad. Sci. USA* 78, 6627-6630.
 39. Yang, J.K., Masters, J.N. and Attardi, G. (1984) *J. Mol. Biol.* 176, 169-187.
 40. Burgeson, R.E., Hebde, P.A., Morris, P.N. and Hollister, D.W. (1982) *J. Biol. Chem.* 257, 7852-7856.
 41. Burgeson, R.E. and Hollister, D.W. (1979) *Biochem. Biophys. Res. Comm.* 87, 1124-1131.
 42. Yamada, Y., Liau, G., Mudryj, M., Obici, S. and de Crombrughe, B. (1984) *Nature* 310, 333-337.