Analysis of cDNA and genomic clones coding for the pro α 1 chain of calf Type II collagen

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ABSTRACT

A bovine cDNA library constructed from fetal cartilage RNA was screened with a pro α 1(II) collagen specific chicken cDNA. A recombinant clone (Bc 7), with an insert of 1 kb, was identified and shown to contain sequences exhibiting 85% homology with the chicken pro α 1(II) collagen C-propeptide. Interspecies comparison strongly suggested that one potential glycosylation site present in the avian C-propeptide is not utilized, since this site is absent in the bovine chain. In addition, two overlapping genomic clones (Pal 3 and Pal 4) were isolated and partially characterized. These clones span 23 kb of DNA and contain approximately 17 kb of the pro α 1(II) calf gene. Sequencing of exon 1 has determined the length of the 3' untranslated region and the exact location of the polyadenylation attachment site.

INTRODUCTION

The fibrillar collagens, Type I, Type II and Type III, constitute the major components of the extracellular matrix of connective tissues, such as bone, skin, tendon, ligaments and cartilage. The biosynthetic precursor molecules (procollagens) contain three major domains: The N- and C-terminal propeptides and a large central triple helical domain. The latter, also called the α chain domain, is characterized by the general formula (Gly-X-Y)_{340±2}. Four distinct loci encode the subunits of the mature collagen types: pro α 1(I), pro α 2(I), pro α 1(II) and pro α 1(III) chains (1).

Type II collagen is the major component of the total tissue collagen in hyaline cartilage, where it provides the supporting network for proteoglycans and other constituents of the extracellular matrix. During and after embryogenesis, the normal process of cartilage differentiation and bone formation depends on the temporal and spatial expression of the Type II and Type I collagen genes (2). *In vitro*, phenotypical dedifferentiation of cultured chondrocytes follows, among other events, the disappearance of Type II collagen and the production of Type I (3-5). A similar biosynthetic "switch" has been invoked in pathological conditions such as osteoarthritis (6).

In order to investigate the regulation of Type II collagen expression in

normal and diseased states as well as to understand the evolution of this gene, several investigators have isolated chicken cDNA and genomic clones bearing Type II collagen sequences (7-12). In addition, some information has also been generated for the rat and human pro α 1(II) gene (13-15). Here we report the isolation and sequencing of a cDNA clone (Bc 7) coding for almost 75% of the C-propeptide domain of bovine Type II collagen. We also have isolated two overlapping genomic clones (Pal 3 and Pal 4), which were found to contain almost 17 kb of the bovine gene.

MATERIALS AND METHODS

cDNA Cloning Procedure

A cDNA library was constructed from size selected poly(A⁺) RNA isolated from the articular cartilage of a 24 week-old calf fetus. Total poly(A⁺) RNA was isolated from frozen tissue according to a modification of previously published procedures (16,17). More precisely, 1 gm of frozen tissue was ground to a fine powder in a blender cooled with liquid nitrogen, resuspended in a solution of 10 mM Tris, pH 8.0, 100 mM EDTA, 1% SDS and 200 μ g/ml proteinase K and incubated at 65°C for 1 hour. Total RNA was then chromatographed on an oligo(dT) cellulose column. The resulting poly(A⁺) RNA was finally enriched for collagen sequences by fractionation on a methyl mercury sucrose gradient. Total poly(A⁺) RNA was prepared from dermal fibroblasts according to the published methodology (17), whereas sterna poly(A⁺) RNA from seventeen day-old chick embryos was a kind gift of Dr. Y. Ninomiya.

The synthesis of double stranded cDNA and oligo(dG) tailing was performed as described (17). Plasmid pBR322 DNA was cleaved with Eco RV and oligo(dC) tailed by terminal transferase (18). Equimolar amounts of plasmid DNA and double stranded cDNA were annealed and used to transform *E. coli* strain MC1061 (19). Bacterial colonies harboring plasmids were selected on ampicillin medium and screened at high density with an *in vitro* labeled cDNA probe (pCs2) coding for the chicken pro α 1(II) collagen (11). The DNA of the positive clones was purified as described (17).

Cell-Free Translation, Nucleic Acid Blotting and DNA Sequencing

Cartilage specific poly(A⁺) RNA enriched for collagen sequences was translated in a rabbit reticulocyte cell-free system and the products of the reaction were analyzed by electrophoresis on a SDS polyacrylamide gel with and without prior treatment by bacterial collagenase as described (20).

Transfer of nucleic acids onto nitrocellulose paper was performed as previously described (17). Cross-hybridizations of the chicken probe to the

bovine RNAs were carried out at 37°C in 5 x SSC (1 x SSC:0.15 M NaCl, 0.015 M Sodium Citrate pH 6.8) 1 x Denhart's solution, 100 µg/ml of sheared salmon sperm DNA and 35% formamide. Washing of the filters was done by sequentially decreasing the salt concentration from 2 x SSC to 0.25 x SSC and by increasing the temperature from 22°C to 55°C. The other hybridizations were carried out at 40°C in 2 x SSC and 50% formamide; washing of the filters was terminated at 0.1 x SSC at 55°C. DNA nucleotide sequencing was performed according to the procedure of Maxam and Gilbert (21). Sequences presented in this paper were based on sequencing of both strands.

Gene Isolation

The calf genomic library in Charon 30 used in these studies was obtained from Dr. D. Capon (Genentech Inc., San Francisco) and contained 10-15 kb inserts of bovine DNA partially digested with the enzyme Sau 3A (22 and Capon, D. et al., manuscript submitted). The screening for the pro α 1(II) collagen genomic clones, their isolation, amplification and DNA purification were performed as described (22,23).

RESULTS AND DISCUSSION

The analysis of the translational products of the RNA purified from calf and chicken cartilages showed an identical pattern of collagenase-sensitive polypeptides (Fig. 1). We therefore based the identification of the bovine pro α 1(II) collagen on similar studies of Upholt et al. (24), who showed that the peptide migrating between the pro α 1(I) and pro α 2(I) chains is indeed pro α 1(II) collagen. The absence of this collagenase-sensitive molecule in the cell-free products of the RNA isolated from adult bovine fibroblasts further substantiated this notion (Fig. 1). Thus we concluded that the poly(A⁺) RNA purified from bovine articular cartilage exhibited a substantial enrichment in Type II mRNA, although some Type I collagen was also present in this tissue. This observation was independently confirmed by Northern blotting hybridizations (Fig. 2). In these experiments the amount of Type I collagen mRNAs relative to Type II appeared to be less than the amount estimated from the translation assay. Conceivably this discrepancy could be the result of the more efficient translation of the Type I mRNAs in the heterologous cell-free system. Alternatively, although the experimental conditions used in the Northern blot analysis were identical, we could not firmly exclude that the assessment of the relative collagen mRNAs content was incorrect because of other variables, such as the different length and/or base compositions of the human Type I probes. Notwithstanding this technical problem, we used the

Figure 1. SDS gel electrophoresis of cell-free translation products of poly(A⁺) RNA purified from bovine adult fibroblasts (Lane 1), chicken fetal sterna (Lane 3) and bovine fetal cartilage (Lane 5). Lanes 2, 4 and 6 are the three translation products digested with bacterial collagenase.

bovine poly(A⁺) RNA to construct a cartilage-specific cDNA library, which was screened with the insert of pCs2(11). The resulting positive clones were amplified, the DNA was purified and in turn used as probe for Northern blot hybridizations of RNA from bovine fetal cartilage and adult fibroblasts.

Figure 2. Northern blotting hybridization of total poly(A⁺) RNA purified from bovine fetal cartilage (Lanes 1,2,3,5) and adult fibroblasts (Lanes 6 and 7) as well as chicken fetal sterna (Lane 4). The probes used were: pCs2 (Lanes 1,4 and 6), Bc 7 (Lanes 5 and 7) and the human Type I clones Hf677 (Lane 2) and Hf32 (Lane 3) (17). Equal amounts of each RNA, 400 ng, were hybridized to the various probes which were *in vitro* ³²P labeled to the same specific activity, approximately 4 × 10⁸ cpm/μg. After hybridization the different filters were autoradiographed for an identical length of time.

Figure 3. Restriction map of Bc 7. The letters indicate the following restriction enzymes: A: Ava I; Ap: Apa I; Av: Ava II; B: Bam HI; E: Eco RI; H: Hha I; K: Kpn I; P: Pst I; X: Xmn I. At the lower right hand corner is the scale expressed in kilobases (kb). The sense of transcription is indicated by the arrow above Bc 7. The jagged line represents the 188 bp of pBR322. The arrows below indicate the fragments sequenced. Also shown is the position of the termination codon UAA.

Using this strategy we were able to assess the size and the amount of the complementary RNA species. With the exception of one recombinant, which was later characterized to code for the pro α 1(I) chain, all of the bovine clones exhibited a pattern in agreement with the size, tissue specificity and quantitative representation predicted for a pro α 1(II) collagen clone (Fig. 2). The DNA from the largest recombinant, Bc 7 (1,011 bp long) was purified, subjected to extensive restriction mapping and sequenced. Figure 3 shows the restriction map of Bc 7 and the approach employed for generating the nucleotide sequences shown in Fig. 4. It should be noted that because of the strategy utilized in the cloning, digestion of Bc 7 with the enzyme Bam HI releases the eucaryotic insert together with 188 bp of the vector DNA stretching from the Eco RV site to the Bam HI site of pBR322 (18).

Since no information is available for the primary structure of the bovine Type II collagen C-propeptide, it was not possible to make a direct comparison between the amino acid sequences derived from Bc 7 and the sequences obtained by protein analysis. There is, however, a high degree of homology in the primary structure of pro α 1(II) chain among different species (14). Nucleotide sequence comparison between Bc 7 and the avian chain (9,10) showed a remarkably high value of similarity (85%) for two species which diverged from each other more than 300 million years ago. Of these changes, 51 were silent, whereas the remaining 31 resulted in 23 amino acid replacements equivalent to an 87% homology. The most significant difference between the two species was the change from a serine to an alanine at position 126. It has previously

been shown that the chicken pro α 1(II) C-propeptide possesses two potential sites for N-linked glycosylation, one located at positions 124 to 126 (Asn-Leu-Ser) and the other at positions 147 to 149 (Asn-Val-Thr) (10). The change observed in the bovine chain conclusively eliminates the formal possibility of two glycosylation sites in the Type II C-propeptide. This is in agreement with evidence showing that the sequence around position 148 is highly conserved in all the fibrillar collagens (25) and with biochemical studies demonstrating the presence of an oligosaccharide side chain in the chicken pro α 1(I) and pro α 2(I) C-propeptides at that position (26).

In conclusion, the data presented here strongly favor the idea that Bc 7 indeed codes for the C-propeptide domain of the pro α 1 chain of Type II collagen. However, one could still argue that the clone might be specific for one of the chains of the minor collagen types found in hyaline cartilage (2). In order to explain the quantitative and qualitative pattern observed in the Northern blotting experiments, the sequence of this minor collagen has to be strikingly similar to that of the major pro α 1(II) chain. Recent studies exclude the possibility that Bc 7 could be Type IX collagen or one of the two collagenous polypeptides 1 α and 2 α (27,28). Although unlikely, the formal possibility remains that Bc 7 represents either Type X collagen (29) or the 3 α chain (28). Several indirect lines of evidence exclude this notion. First, genomic mapping of nuclear DNA by Southern blotting hybridizations strongly suggested that Bc 7 hybridized to a unique locus in the bovine haploid genome (data not shown). Second, screening of a phage library carrying bovine genomic sequences led to the isolation of only two clones which, as discussed later, were found to be derived from the same gene. Third, in parallel investigations we isolated several human genomic fragments whose sequences were similar to those of Bc 7, and matched two known amino acid stretches of the mammalian pro α 1(II) collagen chain (15). With these considerations in mind, for the remainder of this article we shall refer to Bc 7 as a clone specific for the bovine pro α 1(II) collagen chain.

Following the stop codon (UAA), the 3' untranslated region of the bovine pro α 1(II) mRNA extends for 436 nucleotides and ends with a long poly(A) stretch (Fig. 4). Sequencing of the corresponding genomic region demonstrated the post-transcriptional nature of the poly(A) tail of Bc 7 and therefore determined the exact location of the end of the pro α 1(II) mRNA transcript. The canonical polyadenylation signal AUUAAA was found twenty-one bases upstream from the poly(A) tail (Fig. 4) (30). Although the length of the 3' non-coding region of the bovine pro α 1(II) collagen mRNA is very similar to

Figure 4. Nucleotide and amino acid sequences of Bc 7. The amino acid residues are numbered according to Ninomiya et al. (10). Differences with the avian sequences are shown for both nucleotides (above) and amino acids (below). The cysteinyl residues and the canonical sequence for polyadenylation are underlined. The box localizes the carbohydrate attachment site at position 147; the three asterisks emphasize the termination codon for translation.

It is interesting to note that among the four fibrillar collagen genes the Type II is the only one which seems not to be transcribed into multiple mRNAs with length polymorphisms of their 3' untranslated regions (15,31,32). The significance, if any, of this difference is not understood because it has not yet been possible to define a biological function for the collagen polymorphic transcripts (33). Several authors have reported that in Northern blot hybridizations of RNA from chicken cartilage, a larger mRNA species is also present in very small amounts after longer exposure (7,11,12). No data has been thus far presented on the nature of this larger RNA and on its relationship to the predominant 5 kb species. We have not observed the same phenomenon with the bovine articular cartilage or with our preparation of chicken sterna RNA using as probes Bc 7 or pCs2 respectively (Fig. 1 lanes 4 and 5). On the other hand, a minor band of larger size was seen when the bovine cartilage RNA was

Figure 5. Restriction map of the overlapping genomic clones Pal 3 and Pal 4. The letters represent the restriction enzymes found within the 23 kb of DNA: B: Bam HI; E: Eco RI; H: Hind III. Note that the common position of the Eco RI site (middle) is shown. The approximate location of the repetitive sequences is indicated by the gray bars underneath Pal 3 and Pal 4. Exon 1 is represented by the box where black signifies coding elements and white signifies non-coding sequences. The relative position of Pal 3 and Pal 4 within the right (R) and left (L) arms of Charon 30 is shown. The solid vertical bars indicate the Bam HI site restored after the insertion of the Sau 3A digested genomic fragments into the phage arms, whereas the vertical dotted lines indicate the lack of the Bam HI site. Also shown is the sense of transcription of the eucaryotic sequences as well as the scale expressed in kilobases (kb).

cross-hybridized to the chicken Type II probe. Under the same relaxed conditions of hybridization, pCs2 recognized in adult calf fibroblasts RNA the characteristic pattern of the pro α 1(I) collagen, whose larger transcript is of the same size as that of the minor species seen in cartilage (Fig. 1 lane 6). Similarly, when Bc 7 was hybridized to the calf fibroblasts RNA under stringent conditions, the same pro α 1(I) pattern was observed (Fig. 1 lane 7). Thus we concluded that the hybridization of the larger Type II RNA species seen by the other investigators is most likely due to the close sequence homology (82%) which exists between the pro α 1(I) and pro α 1(II) collagens (15,33) and, indirectly, it is an indication of the variable presence of the former species in different RNA preparations from cartilagenous tissues. Further experiments will prove the validity of this hypothesis.

In order to begin to determine the structure of the mammalian pro α 1(II) collagen gene and to compare it to the avian counterpart, 4×10^5 phages harboring bovine genomic sequences were screened with Bc 7 and two clones were identified: Pal 3 and Pal 4 (Fig. 5). The restriction maps of the genomic clones and their orientation with respect to the phage arms were first determined. Because of the strategy utilized in the library construction, Bam HI

Figure 6. Restriction mapping analysis of the genomic clones Pal 3 (B) and Pal 4 (A). In each panel in Lane 1 is the ethidium bromide staining of the phage DNA cleaved with Eco RI and run on an agarose gel. In Lanes 2 and 3 are shown the autoradiographies of the same DNA after Southern blotting hybridization to the 5'- and 3'- specific Bc 7 probes, respectively. The arrows indicate the position of the size markers, which consisted of λ DNA digested with Hind III.

digestion of the phage clones does not always release the eucaryotic insert from the phage arms (22). Thus, DNA from Pal 3 and Pal 4 was digested with the enzyme Eco RI, run on an agarose gel, Southern blotted and hybridized to specific 5' and 3' fragments of Bc 7 (Fig. 6). More precisely, the excised insert of Bc 7 was secondarily restricted with the enzyme Eco RI which yielded a 718 bp long 5'-specific fragment and a 521 bp long 3'-specific fragment. The former hybridized with the 15.5 kb Eco RI fragment of Pal 4 and with the 10 kb Eco RI fragment of Pal 3 (Fig. 6). Only the 15.5 kb segment of Pal 4 was found devoid of phage sequences, whereas the 10 kb fragment of Pal 3 contained 1.1 kb of the left arm of Charon 30. Parallel Southern blotting experiments using total genomic DNA hybridized with the 5' end probe of Bc 7 identified a single band, also 15.5 kb in size (data not shown).

Similar experiments using the 3' end fragment of Bc 7 revealed that this probe hybridized with the 10 kb Eco RI fragment of Pal 4 DNA and the 8 kb Eco RI fragment of Pal 3 (Fig. 6). The latter contained 1.1 kb of DNA from the left arm of Charon 30, whereas the former carried the entire right arm of the

phage vector. Thus, from these mapping experiments, we concluded that, (a) the two clones were derived from the same locus, (b) they overlapped for approximately 10 kb, (c) they covered almost 23 kb of contiguous genomic DNA, of which approximately 17 kb codes for pro α 1(II) sequences and (d) they shared an Eco RI site which is common to the cDNA clone Bc 7 (Fig. 5). These conclusions were further supported by mapping analysis with the enzymes Bam HI and Hind III alone and in combination (Fig. 5). Sequencing of Pal 3 and Pal 4 around the common Eco RI site established their identity with Bc 7. Furthermore, these comparative experiments also defined the exact size of exon 1 (580 bp). One hundred forty-four bases of exon 1 code for a region which spans from amino acid residue 199 to amino acid residue 246 and which contains at position 244 the last cysteinyl residue of the C-propeptide (Figs. 4 and 5). Finally, using nick translated total calf DNA, two repetitive sequences were located within Pal 3 and Pal 4 (Fig. 5).

SUMMARY

In conclusion, we constructed and isolated a cDNA and two overlapping genomic clones containing the 3' end of the calf pro α 1(II) collagen gene. Because of the limited availability of amino acid sequence data, the identity of the clones was based on the high level of homology between the bovine and the avian sequences coding for the C-propeptide domain. Using the calf cDNA we have recently isolated the entire human pro α 1(II) gene (15) and determined its chromosomal location (Huerre, Jeanpierre et al., manuscript submitted). These studies have revealed that the human gene, which is located in the segment 12q131-12q132 of chromosome 12, is significantly larger (30 kb) than its avian counterpart (9). Currently we are defining in more detail the structure of the two mammalian type II collagen genes in order to generate more information about the evolution of this locus. These studies are also an important prerequisite for determining the nature of those factors involved in the physiological and pathological expression of the Type II collagen gene.

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