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Human and Chick α2(I) Collagen mRNA: Comparison of the 5′ End in Osteoblasts and Fibroblasts

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ABSTRACT: Type I collagen, a heterotrimer of two α1(I) chains and one α2(I) chain, is the major structural protein of bone, skin, and tendon. The collagen of patients with bone diseases has been studied in skin fibroblasts instead of osteoblasts because the genes for type I collagen are single-copy genes. While these studies should detect structural changes in the gene, they might fail to detect defects in processes which are dependent on tissue-specific expression. The studies reported here sought to determine whether the expression of type I collagen in skin and bone was characterized by the use of alternate promoters or alternative splicing in the N-propeptide region. Primer extension and nuclease S1 protection experiments were used to analyze the structure of the α2(I) mRNA from the 5′ end of the gene through the N-telopeptide coding region (exons 1–6) in human and chick osteoblasts and fibroblasts. The protection and primer extension experiments using human and chick mRNA demonstrated identical routes of splicing in skin and bone at the first five splice junctions. These studies provide reassurance that information obtained from the study of type I collagen in fibroblasts may be extrapolated to bone.

Type I collagen is the major structural protein of skin, bone, and tendon [for reviews, see Bornstein and Sage (1980) and Prockop et al. (1979a,b)]. The two peptides which comprise the type I heterotrimer, α1(I) and α2(I), are encoded by single-copy genes residing on different chromosomes (Solomon et al., 1984; Hender et al., 1983). Single-copy eukaryotic genes frequently utilize alternate promoters or alternative splicing in different tissues (Young et al., 1981; Sabate et al., 1985). The role these mechanisms may play in achieving tissue-spe-

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cific expression of α1(I) and α2(I) has not been determined.

Modulation of collagen mRNA structure is most likely to occur in the exons at the 5′ and 3′ ends of the gene which code for the N- and C-propeptide extensions, because the exons coding for the mature collagen helix are constrained by the basic Gly-X-Y repeating unit. An excellent candidate for structural rearrangement in the N-propeptide region, for example, is exon 2 which contains two overlapping donor splice sequences and is followed by overlapping donor sequences (Aho et al., 1984).

Evidence for collagen mRNA heterogeneity has been found. In human fibroblasts, Myers found that multiple mRNAs, varying in the length of their 3′ untranslated tails, were transcribed from the α2(I) gene (Myers et al., 1983). Two major species, differing by 200 nucleotides at their 3′ ends, comigrate on gels. The authors raised the possibility that a size difference at the 5′ end of the mRNA might compensate for length discrepancy at the 3′ end. The 5′ end of 2α(I) mRNA was studied in human fibroblasts by Ramirez (Dickson et al., 1985) and in chick calvaria by Boedtker and de Crombrugghe and co-workers (Tate et al., 1983; Vogeli et al., 1981). However, their experiments did not exclude alternate promoters or alternative splicing in the 5′ region.

The purpose of the experiments presented here was to determine whether the expression of the α2(I) collagen gene in osteoblasts and fibroblasts was characterized by the use of alternate promoters or alternative splicing within the region between the 5′ end of the gene and the N-telopeptide coding region (exons 1–6).

We demonstrate here that a single promoter is utilized in both tissues of human and chick. Furthermore, splicing of the exons coding for the N-propeptide region generates a single, identical product in skin and bone.

Materials and Methods

Reagents. [γ-^{32}P]ATP was purchased from New England Nuclear; [α-^{32}P]dCTP was purchased from Amersham Corp. All enzymes were obtained from Bethesda Research Labs or New England BioLabs, except nuclease S1 and reverse transcriptase, which were purchased from Boehringer Mannheim. The pBR322 *Pst*I clockwise primer (16-mer) was purchased from New England BioLabs. The 30-mer, 23-mer, and 20-mer probes were custom-synthesized by OCS Labs of Denton, TX, and the 18-mer probes were kindly synthesized by Millie Schaefer of NHLBI. The chick calvarial cDNA clone pMF21 and the human fibroblast cDNA clone peA21 were kindly provided by Drs. Helga Boedtker and Francesco Ramirez, respectively.

Cells and Tissues. Chick calvarial and long bone tissue was obtained from 16-day chick embryos as previously described (Sobel et al., 1978). Chick embryo fibroblasts (CEF) were cultured from 9–11-day chick embryos (Temin, 1960) and harvested for RNA preparation after the first or second passage.

Human osteoblasts and fibroblasts were obtained from one adult with clinically normal connective tissue who underwent surgery for an unrelated disease. Fibroblasts were grown from skin biopsy in Dulbecco's MEM with 10% fetal calf serum using standard techniques. Osteoblasts were cultured from a rib specimen by Dr. Pamela Robey of NIDR as previously described (Robey & Termine, 1985). Cells were harvested for RNA after the second passage.

RNA Preparation. Total calvarial and long bone RNA was isolated from 16-day chick embryos as previously described (Sobel et al., 1978) and was the generous gift of Dr. Mark Sobel. Total RNA was isolated from cultured chick embryo fibroblasts and human osteoblasts and fibroblasts by the guanidinium isothiocyanate/CsCl method (Chirgwin et al., 1979). Poly(A+)-containing RNA was isolated by chromatography on oligo(dT)–cellulose (type T-3, Collaborative Research) (Aviv & Leder, 1972).

Primer Extension Analysis. The primers were synthetic oligonucleotides complementary to 18 or 30 nucleotides in exon 4 of the cDNA for α2(I) collagen. The 5′ end of each primer was labeled with [γ-^{32}P]ATP using polynucleotide kinase. Approximately 0.3 μg of each primer was hybridized in separate reactions to 2 μg of poly(A+)RNA from fibroblasts and 0.6 μg of poly(A+) RNA from calvaria/osteoblasts in a 40-μL reaction mix containing 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 6.4, 400 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA). The primers were extended (Tate et al., 1983) with reverse transcriptase, and products were electrophoresed on 8% polyacrylamide (PA)/7 M urea gels.

Production of Probes from pMF21 and peA21 for S1 Analysis. The uniformly labeled chick and human single-stranded probes (probe a) were synthesized with Klenow fragment in the presence of [α-^{32}P]dCTP using primers hybridized to plasmid templates which had been digested with the indicated restriction enzyme and then denatured. The end-labeled probes (probes b and c) were made by kinasing appropriate restriction fragments from the cloned cDNA. The restriction fragment strands were separated on neutral 5% PA gels [acrylamide:bis(acrylamide) ratio of 50:1] with dimethyl sulfoxide (DMSO) loading cocktail (Szalay et al., 1977). Both strands were electroeluted for nuclease S1 analysis. Results are shown only from the strand that protected mRNA.

Nuclease S1 Analysis. Uniformly labeled chick probe "a" was individually hybridized in 100 μL to 50 μg of total CEF RNA (46 and 48 °C), 25 μg of total calvarial RNA (48 °C), and 25 μg of tRNA (48 °C) for 3 h in hybridization buffer of 80% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM Pipes, pH 6.4 (Berk & Sharp, 1978). The hybrids were digested with 600 units of nuclease S1 at 42 °C for 30 min. Protected products and full-length probe were sized on an 8% PA/7 M urea gel.

For the experiment shown in Figure 4, panel B1, end-labeled strand-separated chick probe "b" was individually hybridized in 40 μL to 10 μg of total calvarial RNA, 2 μg of CEF poly(A+) RNA with 8 μg of tRNA, or 10 μg of tRNA in hybridization buffer (above) for 3 h at 48 °C. Hybrids were digested with 750 units of nuclease S1 at 42 °C for 30 min. Protected products and undigested probes were sized on an 8% PA/7 M urea gel. For panel B2, probe b was hybridized in 100 μL to 25 μg of total CEF RNA or total calvarial RNA or tRNA for 3 h at 48 °C. Hybrids were digested with 600 units of nuclease S1 and sized as before. The upper band in each lane represents hybridized probe from which the approximately 20-nucleotide dC tail was not digested (Vogt, 1973). This tail can be eliminated under more vigorous S1 conditions.

For the experiment shown in Figure 4, panel C, end-labeled chick probe "c" was hybridized to 25 μg of total CEF RNA or tRNA at 48 °C for 3 h in 100 μL. Hybrids were digested with 600 units of nuclease S1 for 30 min at 42 °C, and products were sized as above.

For the human RNA analyzed in Figure 5, uniformly labeled probe or end-labeled *Apa*I/*Nhe*I fragment was hybridized in 25 μL to 10 μg of either total osteoblast RNA or total fibroblast RNA with 15 μg of tRNA in hybridization buffer for 3 h at 48 °C. Hybrids were digested with 750 units

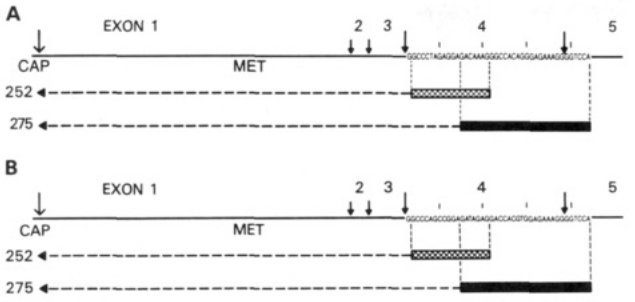

FIGURE 1: 5′ end of α2(I) collagen mRNA. (A) Schematic of experimental plan used for primer extension at the 5′ end of chick α2(I) collagen mRNA. Collagen mRNA from the transcription start site through exon 6 is shown on the top line. the 18-mer (cross-hatched bar) and 30-mer (solid bar) primers are shown, along with the primer extension products expected from their hybridization to exon 4 and extension to the known 5′ end of the α2(I) message. (B) Schematic of experimental plan used primer for extension at the 5′ end of human α2(I) collagen mRNA. Symbols as in panel A.

of nuclease S1, and products were sized as for the chick experiments.

RESULTS

Comparison of Bone and Fibroblast mRNA by Primer Extension. The 5′ end of the α2(I) mRNA was first mapped by primer extension. A pair of synthetic primers was used for both the chick and human experiments (Figure 1). The first primer is an 18-mer complementary to the 5′ end of exon 4; the second is a 30-mer that overlaps the first primer by seven nucleotides and extends farther 3′ through the remainder of exon 4 and into the 5′ end of exon 5 (see Figure 1). Each primer was extended to a common end or ends. Primer extension products that were 23 nucleotides longer when primed by the 30-mer than by the 18-mer were considered to have originated in exon 4.

Figure 2 shows that, in both chick (panel A) and human (panel B) systems, fibroblasts and osteoblasts have a common 5′ terminus resulting in paired primer extension products of 275 and 252 nucleotides (nt). The sizes of these products correspond exactly to those predicted for extension to the known 5′ end of α2(I). The 252-nt product from chick calvarial mRNA was eluted from the gel and subjected to Maxam–Gilbert sequencing. The sequences obtained were identical with the published sequences (Tate et al., 1983) for the 5′ end of chick α2(I) message. Primer extension using mRNA from chick crop, a mature tissue, gave a series of products identical with CEF.

In primer extensions using the human mRNAs, only paired products corresponding to extension to the known end were seen with fibroblasts mRNA. The longest product seen with the 30-mer primer and human osteoblast mRNA also corresponds with extension to the known 5′ end. In the chick system, multiple minor smaller products were seen even under stringent conditions (Figure 2, panel A, and legend). The arrangement of the minor calvarial bands suggests that they do not represent the same 5′ end. Furthermore, S1 protection experiments (see below) suggest that these products were not primed from the 5′ end of α2(I) mRNA.

The paired chick calvarial primer extension products at 205 nt/228 nt were quite prominent in some mRNA preparations. Partial sequences were obtained from the calvarial 205-nt product; they did not correspond to exons 1, 2, or 3 of the known cDNA or their introns. Oligonucleotides corresponding to portions of the 205-nt extension did not hybridize in Southern blots (data not shown) to cloned chick genomic DNA extending 10 kilobases (kb) upstream of the known transcription start (genomic clones kindly provided by B. de

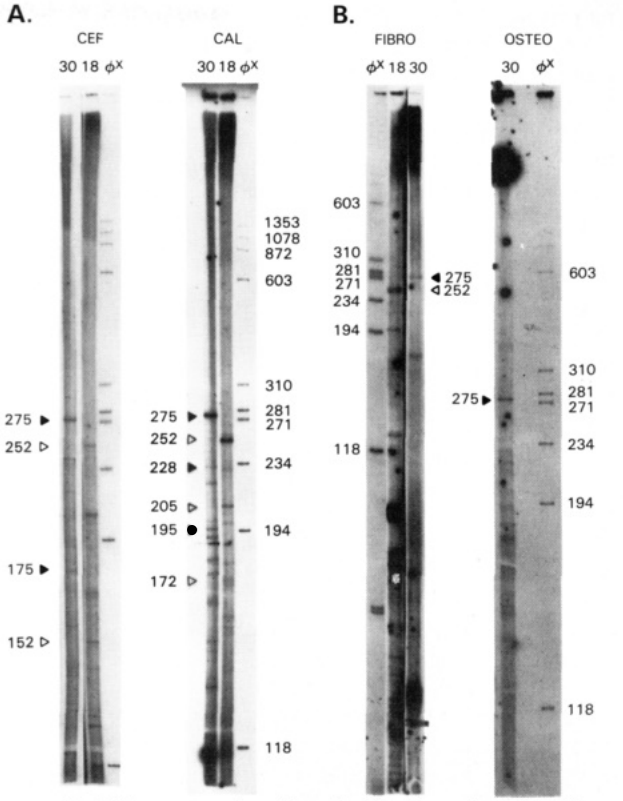

FIGURE 2: Primer extension of synthetic oligonucleotide primers hybridized to chick (panel A) or human (panel B) poly(A+) RNA. Primer extension products were sized on an 8% PA/7 M urea gel. Products from 30-mer extension are indicated as solid arrowheads and those from 18-mer extension as open arrowheads. *Hae*III restriction fragments of φX174 are shown on each gel as size markers. Minor paired chick products are indicated: in CEF, 152 nt (18-mer)/175 nt (30-mer); in calvarial mRNA, a cluster of bands around 170 nt (18-mer) and a discrete band at 195 nt (30-mer). The different arrangements of these bands suggests that they do not represent the same 5′ end.

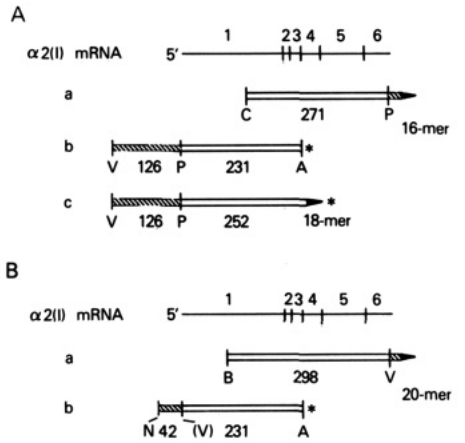

FIGURE 3: Schematic of probes used for nuclease S1 protection analysis and their alignment with the known 5′ end of α2(I) cDNA. Panel A shows the structure of probes from pMF21, used for chick S1 nuclease experients. Panel B shows the structure of probes from peA21, used for human nuclease S1 experiments. Probe regions complementary to chick or human α2(I) cDNA insert are shown as open bars; probe regions complementary to pBR322 are depicted as hatched bars; oligonucleotide primers are shown as solid arrowheads; label positions for end-labeled probes are depicted with asterisks. Restriction enzyme sites are indicated as follows: (for panel A) C, *Acc*I; P, *Pst*I; V, *Pvu*I; A, *Apa*I; (for panel B) B, *Bst*EII; N, *Nhe*I; A, *Apa*I; V, *Bam*HI (generated from *Eco*RV site during cloning); (V), unregenerated *Eco*RV site.

Crombrugghe). In Northern blots using chick calvarial and CEF poly(A+) RNA, these oligonucleotides hybridized to

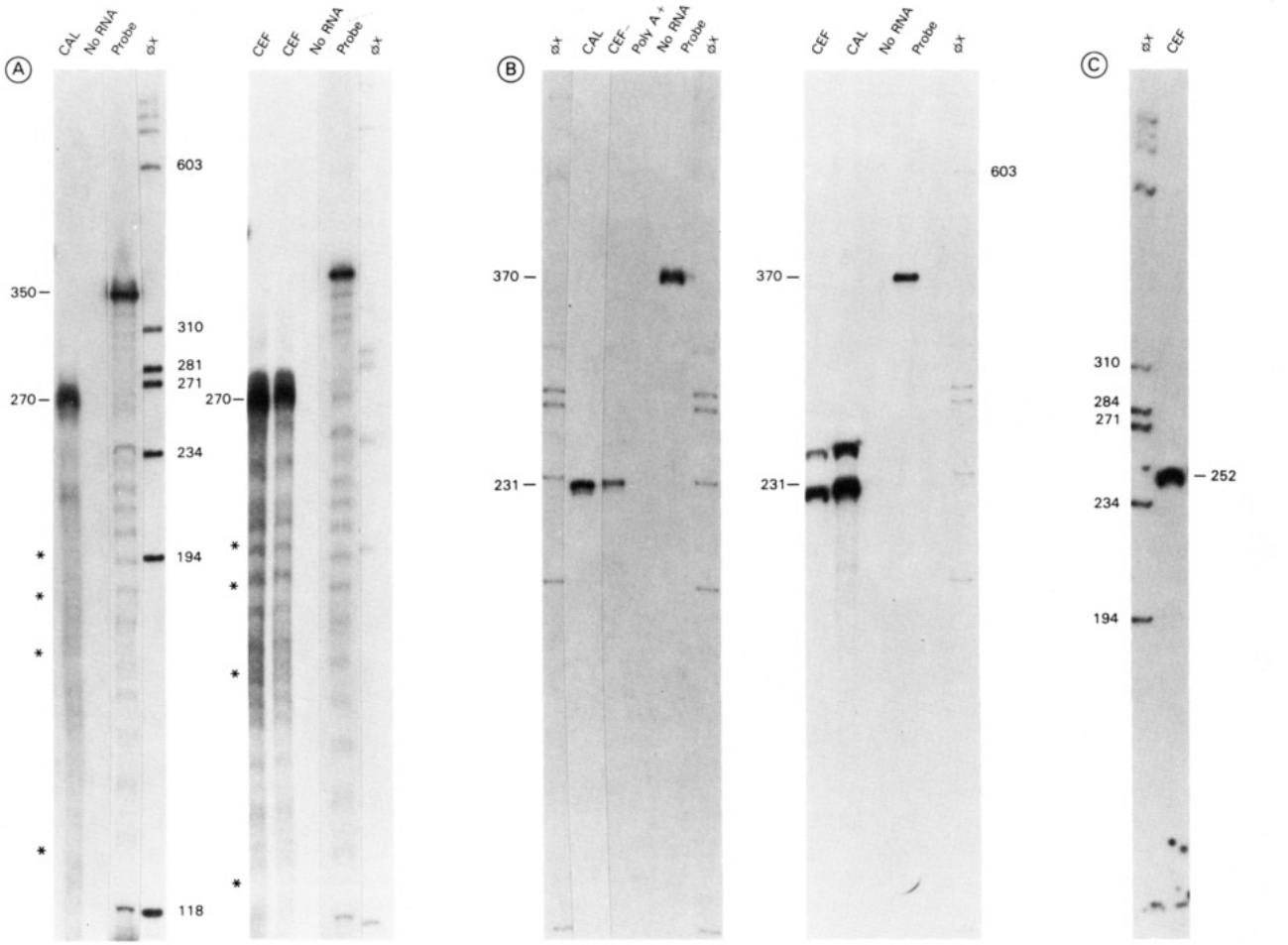

FIGURE 4: Nuclease S1 protection analysis of chick α2(I) mRNA. Experiments shown in panels A, B, and C utilized probes a, b, and c, respectively, from Figure 3, panel A. All protection products were sized on 8% PA/7 M urea gels with *Hae*III restriction fragments of ϕX174 used as markers. Probes were hybridized at the temperature indicated below under the conditions described under Materials and Methods and digested with nuclease S1 as described. (A) Probe a, about 350 nt in length [complementary to 271 nt of α2(I) calvarial cDNA, about 25 nt of homopolymer dG tail, and 53 nt of pBR322], was hybridized (left gel) to 25 μg of total calvarial RNA or 25 μg of tRNA at 48 °C or (right gel) to 25 μg of total CEF RNA at 46 or 48 °C or 25 μg of tRNA at 48 °C. The full-length probe and protected products corresponding to the known 5′ end of α2(I) are indicated. Chick probe a was a uniformly labeled probe and, on the overexpossd film shown, is subject to the natural breakdown of such probes. Fragments of the sizes expected from alternative splicing (asterisks) were not seen. (B) Probe b, 370 nt in length and complementary to 231 nt of α2(I) calvarial cDNA, was hybridized to (left gel) 10 μg of total calvarial RNA, or 2 μg of CEF poly(A+) RNA, or 25 μg of tRNA, all at 48 °C, or to (right gel) 25 μg each of total CEF RNA, total calvarial RNA, or tRNA at 48 °C. The full-length probe and protected products corresponding to the known 5′ end are indicated. The upper bands in CEF and calvarial protection lanes, in the gel to the right, are due to incomplete digestion of the homopolymer dC tail from hybridized probe (Vogt, 1973). This tail can be eliminated under more vigorous S1 conditions. (C) Probe c, 378 nt in length and complementary to 252 nt of calvarial α2(I) cDNA, was hybridized to 25 μg of tRNA or total CEF RNA at 48 °C. The size of the protected product corresponding to the known calvarial 5′ end is indicated.

RNA comigrating with 18S rRNA using conditions under which the 18-mer and 30-mer primers hybridize to α2(I) size message at 4.7 kb. Therefore, we conclude that these products do not represent alternatively spliced mRNA.

The primer extension data suggest that the α2(I) mRNA has a common promoter in bone and skin tissues and provide no support for alternative splicing in the N-propeptide region.

S1 Protection Mapping of the 5′ End of Chick α2(I) mRNA. The structure of the 5′ end of chick fibroblast and calvarial α2(I) mRNA was further studied by nuclease S1 protection experiments (Berk & Sharp, 1978). Three probes covering the first five splice junctions were used (Figure 3A): Probe a extends from the middle of exon 1 into exon 6, probe b is complementary to the region from the transcription start site to the end of exon 3, and probe c is the same as probe b with an extension covering the third splice junction. The letter designation of each probe in Figure 3A corresponds to the letter designation on the Figure 4 panel showing the results of the experiments using that probe.

The three probes were individually hybridized to chick CEF and calvarial mRNA. The relative intensity of the protected products was proportional to the amount of mRNA used in each hybridization. The major fragments protected by the probes were identical in the two tissues in each case (Figure 4). This demonstrates that the major route of splicing is identical in the two tissues at the first five splice junctions.

The data were carefully examined for products of possible minor alternative splicing pathways. The end-labeled probes b and c provide strong evidence that no alternative splicing is occurring. Only the full protected product is seen in both CEF and calvarial systems. The upper band in panel B2 (Figure 2) is about 20 nt longer than the protected calvarial α2(I) 5′ end. It represents the oligo(dC) portion of the probe and was seen when lower S1:probe ratios were used because of the relative resistance of dC homopolymers to S1 digestion (Vogt, 1973). On long overexposure of the calvarial and CEF poly(A+)-protected products, minor bands are seen at 184 and 203 nt in each digest; they comprise less than 5% of the total product on a densitometric scan. Both of these products are too long to represent a minor alternative splicing pathway; each would end within the first 40 nt of the 5′ end of exon 1. They may represent a fortuitous site of mRNA degradation.

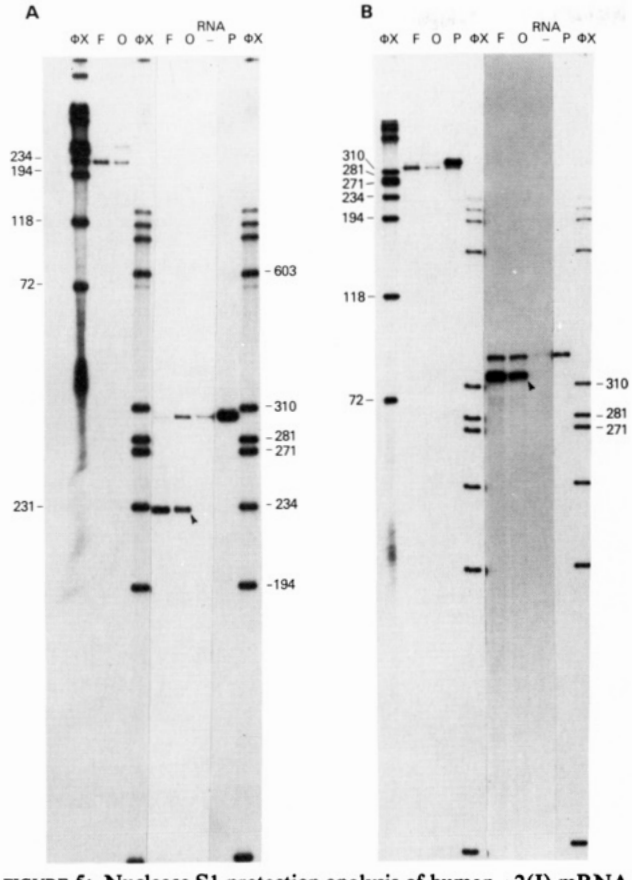

FIGURE 5: Nuclease S1 protection analysis of human α2(I) mRNA. The protected products were sized on 8% PA/7 M urea gels with *Hae*III fragments of ϕX174 used as markers. Reaction products and markers were loaded a second time (lanes on left side of each panel) to allow visualization of smaller products. Symbols used were the following: F, fibroblast RNA; O, osteoblast RNA; RNA–, only tRNA added to probe; P, probe alone; ϕX, *Hae*III fragments of ϕX174; solid arrowheads, protected product. (A) This panel utilized the end-labeled probe b shown in Figure 3, panel B. This single-stranded probe, complementary to 231 nucleotides of α2(I) cDNA and 42 nucleotides of pBR322, was hybridized to osteoblast and fibroblast RNA as described under Materials and Methods. The 231-nucleotide protected product corresponding to the known cDNA 5′ end is indicated. (B) These experiments utilized the uniformly labeled probe a shown in Figure 3, panel B. It is a single-stranded probe complementary to 298 nucleotides of cDNA sequences, about 20 nucleotides of homopolymers dG tail, and 22 nucleotides of pBR322. When this probe was hybridized to osteoblast and fibroblast RNA as described under Materials and Methods, the protected product corresponds in length to the full length of cDNA in the probe plus the 20 nucleotides of S1-resistant dC tailing.

The S1 protection experiments on the 5′ end of CEF and calvarial mRNA collectively provide strong evidence that the two tissues are processing the 5′ end of the α2(I) message identically and that no minor routes of alternative splicing are utilized.

S1 Protection Mapping of the 5′ End of Human α2(I) mRNA. The S1 protection experiments on the human α2(I) mRNA from osteoblasts and fibroblasts used two probes analogous to those in the chick S1 experiments (Figure 3B). The first probe covered the middle of exon 1 through the middle of exon 6; the second probe covered the transcription start site through the 3′ end of the exon 3.

These probes were each hybridized to mRNA from human fibroblasts and osteoblasts; the fragments protected from S1 nuclease digestion are shown in Figure 5. For each probe, a single major band is protected, and that protected fragment is identical in the two tissues. In panel A of Figure 5, the major product corresponds in length to the full length of cDNA in the probe plus 20 nucleotides of S1-resistant dC tailing. Even on long overexposure of the gel, only a few faint minor bands are seen. On overexposure of the gel in panel B (data not shown), the only minor bands seen are about 20 nucleotides longer than the protected product; they can be attributed to the dC homopolymer region of the probe. These experiments clearly demonstrate that a single promoter is used by the α2(I) gene in both human osteoblasts and fibroblasts and that there are no quantitatively significant routes of alternative splicing in the N-propeptide region of the mRNA isolated from these two tissues.

Discussion

The studies presented here on the 5′ end of α2(I) collagen mRNA in human and chick represent the first comparative analysis of promoter usage and RNA splicing in skin and bone, the dominant tissues of type I collagen expression. Using primer extension and nuclease S1 protection, we have found that the α2(I) gene utilizes a single promoter in skin and bone. Also, splicing from the transcription start site through the region coding for the N-telopeptide generates a single product which is identical in both tissues.

The absence of alternative splicing among the known exons is shown by the S1 protection data. The absence of an alternative upstream transcription start site is demonstrated by the combination of the primer extension and S1 protection experiments. Primer extension, using the paired probes shown in Figure 1, in every case corresponds to extension to the known 5′ end. Because the primers used hybridize to exon 4, any transcript initiating upstream of the known start would be detected provided that such a transcript included exon 4. Second, the S1 protection data demonstrate that the known exons 1–6 are never found alternatively spliced; in particular, no transcripts are found that include other exons in the known N-propeptide end but do not include exon 4. The only possibility left open by the combination of these experiments is of an entire alternative set of exons replacing the known exon 1–5 coding region. Excluding this formal possibility would require searching mRNA transcripts with upstream genomic probes.

Two alternative structures at the 5′ end of the α2(I) message have been described. In mRNA from mouse skin, tail, and 3T3 cells, a second transcript has been detected that is colinear with the known transcription product and 100 nt shorter at the 5′ end (A. Schmidt and B. de Crombrugghe, personal communication). We do not believe that we have missed such a shorter transcription product since our S1 protection experiments (1) were done with the identical hybridization cocktail over the same temperature range and (2) would have generated a protected region that was more stable since our probes were complementary to an additional 50 nt in the 3′ direction.

In chick chondrocytes, Adams and co-workers have demonstrated that the nontranslatable α2(I) message may be structurally altered at the 5′ end, involving at least the sequences of exon 1 (personal communication). Such a mechanism does not appear to be used by tissues in which type I collagen is abundantly expressed.

Control of the expression of type I collagen, one of the most abundant and ubiquitous vertebrate proteins, is gradually being revealed as a highly complex affair. This work shows that tissue-specific control in expressing tissues is not achieved through the use of alternate promoters or alternative splicing at the 5′ end. Control may reside at other levels, such as trans-acting factors for transcriptional enhancement or translational repression.

A strong motivation for these studies is the reliance placed on dermal fibroblasts for protein and molecular genetic studies of heritable connective tissue disorders which involve primary bone manifestations, such as osteogenesis imperfecta. Dermal fibroblasts are utilized because of their relative availability and ease of maintenance in culture. Since the genes of type I collagen are single-copy genes, studies using fibroblasts should detect mutations in the portions of the genes coding for procollagen chains. However, there remains the concern that defects in processes which are dependent on tissue-specific expression in osteoblasts would not be detected in patients' fibroblasts. Our studies demonstrate that the $\alpha 2$(I) gene utilizes a single promoter and single set of exons in the N-propeptide region of human primary osteoblasts and fibroblasts. Thus, the data presented here provide the first justification at the RNA level for the extrapolation to bone tissue of information obtained from the study of type I collagen genes and protein products in cultured fibroblasts.

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