Quantitative Analysis of Collagen Expression in Embryonic Chick Chondrocytes Having Different Developmental Fates*

Louis C. Gerstenfeld‡, Mitchell H. Finer§, and Helga Boedtker¶

From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138 and the ‡Laboratory for the Study of Skeletal Disorders and Rehabilitation, The Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

A quantitative determination of collagen expression was carried out in cultured chondrocytes obtained from a tissue that undergoes endochondral bone replacement (ventral vertebra) and one that does not (caudal sternum). The "short chain" collagen, type I1, is only expressed in the former while the other "short chain" collagen type IX, was primarily expressed in the latter. These two tissues also differ in that vertebral chondrocytes express moderate levels of both type I procollagen mRNAs which were translated into full length procollagen chains both in vivo and in vitro, while caudal sternal chondrocytes did not. The percent of collagen synthesis was about 50% in both cell types, but sternal cells expressed twice as much collagen as vertebral cells even though type II procollagen was more efficiently processed to α-chains in vertebral chondrocytes than in sternal chondrocytes.

The number of type II procollagen mRNA molecules/cell was found to be about 2300 in vertebral chondrocytes and about 8000 in sternal cells, in good agreement with the results reported by Kravis and Upholt (Kravis, D., and Upholt, W. B. (1985) Dev. Biol. 108, 164–172). There were about 630 copies of type I procollagen mRNAs with an a1/a2 ratio of 1.6 in vertebral chondrocytes compared with 5100 copies and an a1/a2 ratio of 2.2 in osteoblasts, and less than 40 copies in sternal cells. Since the rate of type I collagen chain synthesis was 50 times greater in osteoblasts than in vertebral cells, type I procollagen mRNAs were about six times less efficiently translated in vertebral cells than in osteoblasts. The type I mRNAs in vertebral chondrocytes were polyadenylated and had 5' ends that were identical in osteoblasts, fibroblasts, and myoblasts. Moreover, type I mRNAs isolated from vertebral chondrocytes were translated into full length preprocollagen chains in vitro in rabbit reticulocyte lysates. Thus, chondrocytes isolated from cartilage tissues with different developmental fates differed quantitatively and qualitatively in total collagen synthesis, procollagen processing, and distribution of collagen types.

Chondrocytes which undergo endochondral bone replacement and chondrocytes which remain as hyaline cartilage have been shown to differ qualitatively and quantitatively in the expression of two "short chain" collagens, type X and type IX. These collagens are smaller than the fibrillar collagens (less than 100 kDa) and have been shown to be unique to cartilage (Mayne and Irwin, 1986). Type X has been immunologically localized to cartilage undergoing hypertrophic endochondral replacement (Schmid and Linsenmayer, 1985; Capasso et al., 1984; Gibson et al., 1982). Organ and cell culture studies have demonstrated that type X collagen was only expressed by hypertrophic cells (Reginato et al., 1986; Ninomiya et al., 1986; Jimenez et al., 1986; Gibson and Flint, 1985; Habuchi et al., 1985; Schmid and Linsenmayer, 1983), while type IX collagen was expressed primarily, but not exclusively, in tissues remaining as hyaline cartilage (Ninomiya et al., 1986; Reginato et al., 1986; Wu and Eyre, 1984).

In order to further characterize the phenotype of chondrocytes isolated from two different cartilage tissues which have different developmental fates, a quantitative study was made of total collagen synthesis, type II and type I collagen synthesis, type II procollagen processing, and the steady state levels of type I and II procollagen mRNAs in caudal sternal chondrocytes, a tissue which remains as hyaline cartilage, and in ventral vertebral chondrocytes, a tissue which undergoes endochondral replacement. The most striking difference between these two cell types is that vertebral but not sternal cells have moderate levels of type I procollagen mRNAs which are translated into type I procollagen chains both in vivo and in vitro.

MATERIALS AND METHODS

Sternal and Vertebral Chondrocyte Cell Cultures—Chondrocytes were prepared from the caudal half of 16-day-old chicken embryo sternum and from the ventral half of 12-day-old chicken embryo vertebra as described previously (Finer et al., 1985). Identical culture conditions and periods of time of cell growth in culture were used for both chondrocyte cultures. Cells were initially grown in minimum essential medium for a 7-day period and only floating, nonattached cells were used for subcultivation. Cells were subcultivated into Dulbecco's modified essential medium, and greater than 95% of the sternal cells remained as floating cells after 72 h while the vertebral cells all attached. All experiments were performed 7 days after the subcultivation. Myoblasts were prepared as described by Gerstenfeld et al. (1984) and cultured in the presence of 25 μg/ml ovotransferrin and 10% fetal bovine serum for 7 days. Osteoblasts were prepared as described by Gerstenfeld et al. (1987) and after subcultivation, day 3 cultures were used for RNA extraction and pulse labeling. Tendon fibroblasts were prepared as described by Schwartz et al. (1976) and cultured in the presence of 25 μg/ml ascorbate and 10% bovine serum for 12 days.

Protein Pulse-labeling, Cell-free Translation, and Protein Analysis— Cultures were labeled for 1 h using 5 ml of Dulbecco's modified

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† Present address: The Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142.

‡ To whom correspondence should be addressed.
essential media supplemented with 250 μCi of [H]proline (110 mCi/ 
mmol) as described previously (Gerstenfeld et al., 1983). After the 1-
h pulse, media was removed, cell layers were washed with phosphate-
buffered saline, and 5 ml of serum-free Dulbecco’s modified 
eductive material was added. Chase time points were 0.5, 1.25, 6, and 24 h after 
the 1h pulse. Media was removed, the cell layers were proteolytically 
acted using 6 μg manidine HCl (Gerstenfeld et al., 1984), and 
radioabeled type I procollagen protein was prepared from osteoblast 
cells as described by Gerstenfeld et al. (1987). Pepsin treatment 
of chondrocyte media was carried out by resuspending 500,000 
cpms of nondialyzable labeled protein in 5 ml of 5% (v/v) acetic acid 
and digesting the protein with 10 pg/ml pepsin at 4 °C for 24 h. Samples were dialyzed against distilled water until a neutral pH was 
reached, and then lyophilized. 76,000 cpm were used/ lane for each 
gel.

For indirect immunoprecipitation, labeled proteins (100,000 cpm 
total radiolabeled cell extracts for osteoblast cultures and 750,000 
cpm for vertebral cultures) were adjusted to 0.5 ml of 150 mM NaCl, 
50 mM Tris-HCl, pH 7.6. Insoluble material was removed by centrif-
gination at 12,000 rpm for 1 min. The supernatants were reacted at 
22°C for 90 min with rabbit antiserum against type I procollagen (Linsenmayer et al., 1982). Indirect immunoprecipitation resulted 
following addition of goat anti-rabbit antibodies during the subsequent 
2-h incubation. The precipitates were collected through a 1 μm sucrose 
cushion and washed as described previously (Sonenshein et al., 1975).

Collagen peptides were identified by digesting proline-labeled protein 
(50,000 cpm) with collagenase, and the amount of collagenase-sensi-
tive material was determined by the method of Peterkofsky and 
Diegelmann (1971). CNBr peptide mapping of collagen was carried out 
as described by Gerstenfeld et al. (1983) with comparison to the 
previously published data (Bornstein and Sage, 1980) and for type X collagen (Regnato et al., 1986; Schmid and 
Conrad, 1982).

mRNA was translated in rabbit reticulocyte lysates prepared by the 
method of Lodish and Nathan (1972) and treated with micrococcal 
nuclease (Pelham and Jackson, 1976). Lysates were optimized for the 
translation of procollagen mRNA as previously described (Gersten-
feld et al., 1983) using [H]leucine as the label. Proteins were analyzed 
by electrophoresis on 5-10% continuous gradient sodium dodecyl 
sulfate (SDS)-polyacrylamide gels (Laemmli, 1970). Fluorography 
was carried out by the method of Bonner and Laskey (1974) and 
Laskey and Mills (1975). The gels were dried and exposed to pre-
flushed x-ray film from 3 to 7 days at -50 °C. Quantitation of the 
fluorographs was performed on a LBK Ultrascan II densitometer 
(LKB Bromma, Sweden).

Analysis of Procollagen mRNA Expression—Total RNA was ex-
tracted from embryonic calvaria and chondrocytes using a modifica-
tion of the phenol-proteinase K method (Gerstenfeld et al., 1983).
Polyadenylated mRNA was prepared as described by Tate et al. (1985) 
and labeled with [32P]ATP. 364-base pair Aua-Sau3A fragment for a2(I) prepared from pMF2l (Young et al., 1984); a 380-base pair 
Bam-Sal fragment for al(I1) collagen probe (Ninomiya and Olsen, 1984), and type 
Al collagen probe (Regnato et al., 1986; Schmid and 
Conrad, 1982).

The abbreviations used are: SDS, sodium dodecyl sulfate; Pipes, 
1,4-piperazinediethanesulfonic acid.
mRNA using a 14-mer corresponding to the sequence starting at the position of the Acl restriction site, depicted in Fig. 5A. The expected extension product of the α1(I) primer is 93 base pairs. The sequence used for primer extension of proc2(I) mRNA was a 15-mer corresponding to the first 15 bases of exon 3 of this gene. The expected size of α2(I) extension is 229 base pairs. Primer hybridization and cDNA synthesis were carried out as described by Tate et al. (1985).

RESULTS

Collagen Synthesis and Processing—Total collagen synthesis was examined in vertebral and sternal chondrocyte cultures. A summary of these results presented in Table I indicates that sternal chondrocytes synthesized approximately two times more collagen/cell than vertebral chondrocytes. Both types of chondrocytes, however, devoted a very high percentage of their protein synthesis to collagen production, with sternal cells devoting a slightly higher percentage, 56%, compared with 50% for vertebral cells. The variation in distribution of the newly synthesized collagen between the cell layer and media compartments, shown in Table I, reflects the physical differences in the growth patterns between the two culture systems. Sternal cells grew as nonadherent floating cells while vertebral cells grew as adherent polygonal cells. Thus, most of the newly synthesized collagen of the vertebral cultures was secreted and incorporated into the matrix of the cell layers. In contrast, the floating sternal cells secreted their collagen into a gelatinous matrix which forms in the culture media, but upon separation of the cells from the media by centrifugation, the newly synthesized collagen was left in the supernatant while the cells were pelleted.

The analysis of the newly synthesized collagen proteins in sternal and vertebral cells is shown in Fig. 1A. This analysis compared the proteins isolated from the cell layers, the media, and the pepsin-treated media of sternal and vertebral chondrocyte cell cultures pulsed for 1 h followed by a 2.5-h chase. A prominent proc1(II) collagen band is present in the profiles of both vertebral and sternal cell extracts, with only minor differences detected in the profiles of newly synthesized intracellular proteins (Fig. 1A, left panel). Proc1(II) collagen appeared to be more rapidly processed in vertebral cells than in sternal cells. The identification of the processing intermediates was identical to that made by Schmid and Conrad (1982) and was verified by cyanogen bromide peptide mapping of each of the identified bands. Two other prominent proteins were found in the vertebral but not in the sternal chondrocyte profiles. One was a noncollagenous protein of approximately 220 kDa and the other was a collagenase sensitive protein of about 60 kDa. These proteins were identified as fibronectin and type X collagen based on their size and previous identification (Schmid and Conrad, 1982; Adams et al., 1982; Gerstenfeld et al., 1985). Scanning densitometry of the media protein profiles indicated that the 60-kDa collagen comprised 40% of the total collagenous media proteins.

In comparison, two collagenase-sensitive bands of 85 and 70 kDa were seen in profiles of the sternal media proteins but not in the vertebral media profiles. These correspond to the α3- and α1-chains of type IX collagen. This identification was based on these proteins' collagennous nature, their reported molecular weight (Mayne et al., 1984; von der Mark et al., 1984; van der Rest et al., 1985), and their chromatographic behavior on DEAE-cellulose, which was consistent with that reported by von der Mark et al. (1984). Scanning densitometry of the media profiles indicated that the 85 and 70 kDa collagens constituted about 12% of the sternal culture media collagen.

Table I

| Collagen Synthesis in Vertebral and Sternal Chondrocytes | Total cpm | Total %Collagen |
|---------------------------------------------------------|----------|----------------|
| Vertebral Cell Layer                                    | 4.2 × 10⁴ | 50             |
| Vertebral Media                                         | 2.1 × 10⁴ | 67             |
| Vertebral Pepsin                                        | 0.9 × 10⁴ | 36             |
| Sternal Cell Layer                                      | 7.7 × 10⁴ | 56             |
| Sternal Media                                           | 4.3 × 10⁴ | 7              |
| Sternal Pepsin                                          | 4.0 × 10⁴ | 93             |

*Protein synthesis and μg of DNA/100-mm dishes were determined from an average of three separate preparations from cells labeled for 24 h. Each determination was from at least triplicate samples. Total cpm and percent of collagen were calculated using the formula of Peterkofsky and Diegelmann (1970) as modified by Schwartz et al. (1976), and the corrected values for cpm of collagen are reported in the table. All measurements are expressed as the mean result and no measurements had a range greater than 18%.

Fig. 1. SDS-polyacrylamide gel electrophoresis of [*H]proline pulse-labeled proteins, synthesized by vertebral and sternal chondrocytes. A, vertebral (V) and sternal (S) cell cultures were pulse labeled for 1 h followed by a 2.5-h chase. Cell layer, media and pepsin-digested media proteins were extracted as described under "Materials and Methods." 75,000 cpm of each sample were electrophoresed on a 5–10% SDS-polyacrylamide gradient gel. The gel was exposed to preflashed film for 3 days. FN, fibronectin; pro, unprocessed procollagen; pN and pC, partially processed procollagen retaining the amino-terminal or carboxy-terminal propeptide, respectively; 60K, type X collagen, formerly called short chain collagen; arrows identify collagenase-sensitive polypeptides of 85 and 70 kDa believed to be the α3 and α1 chains of type IX collagen; O, osteoblast control. B, immunoprecipitation of [*H]proline pulse-labeled protein with type I antisera. V, vertebral and O, osteoblast media proteins were used for the immunoprecipitation experiments.

Pepsin digestion of the media proteins from 24-h labeled samples was carried out to remove noncollagenous proteins from the media proteins and to verify the identification of the collagen bands. The sternal sample contained a single major pepsin-resistant protein corresponding to the α1(II) chain, while the vertebral sample contained two prominent pepsin-resistant species the α1(II) collagen band and one corresponding to a 45-kDa pepsin-resistant protein (Fig. 1A, right panel). The reduction of type X chain from 60 to 45 kDa following pepsin treatment has been reported previously (Schmid and Linsenmayer, 1983). Upon longer fluorographic exposure of...
the sternal lanes, additional pepsin fragments of 50, 35, 30, and 20 kDa were detected, presumably derived from type IX collagen chains.

It was not possible to positively identify either proα1(I) and proα2(I) or the α(I) collagen chains in the cell layer and media profiles in Fig. 1A, indicating that if type I collagen was present, it was being synthesized at very low levels. A minor band with the mobility of α2(I) chains, however, could be seen in the pepsin-digested media from vertebral cells. The ratio of the α2(I) to the α1(I+II) was 1:2.4. Assuming that the ratio of α1(I) to α2(I) is 2:1, the ratio of type II to type I collagen chains in vertebral cells is 2:3, that is, the rate of type II chain synthesis is about seven times greater than that of type I. However, since pepsin may preferentially digest α2(I) collagen, this may be an underestimation of the amount of type I collagen.

To confirm the pepsin results, indirect immunoprecipitation of the radiolabeled type I collagen was carried out. Proteins synthesized by either vertebral chondrocytes or day 3 osteoblasts (used as control) were precipitated with antisera to type I collagen (Linsenmayer et al., 1982) and then examined by polyacrylamide gel electrophoresis. These results are shown in Fig. 1B. The vertebral and osteoblast samples both contain the α1 and α2 chains of type I collagen, but the osteoblast sample also had proα1(Ι) and proα2(Ι) chains. It was necessary to use eight times more radiolabeled protein to obtain the barely detectable immunoprecipitated products from the vertebral samples. The gel profiles were scanned, and the osteoblast sample had almost seven times more labeled type I collagen chains than the vertebral sample. Since the cpm could be normalized to DNA and the total cpm/immunoprecipitation was known, it was calculated that there was roughly 50 times more synthesis of type I chains/osteoblast cell than per vertebral cell.

Either short term pulse labeling, shown in Fig. 1, or steady state 24-h labeling indicated that more processed α1(Ι) molecules had accumulated in the vertebral cultures than in sternal cultures. These results would suggest that vertebral cultures processed their collagen more efficiently than sternal cultures. In order to examine this possibility, a pulse chase experiment was carried out. These results, shown in Fig. 2A, clearly demonstrate that the vertebral cultures converted their procollagen to α-chains, while sternal cultures did not. Moreover, sternal cells secrete collagen at a slower rate than vertebral cells as indicated by the lower amounts of labeled protein seen in the culture media at earlier time points. Analysis of the kinetics by scanning densitometry (Fig. 2B) demonstrated that the amino-terminal propeptide was processed more efficiently than the carboxyl-terminal propeptide with an overall t½ of >6 h for vertebral cultures. However, in sternal cultures less than 20% of the proα1α-chains were converted to the processing intermediates after 24 h. As expected, neither the 60-kDa collagen species in the vertebral cultures nor the 85- and 70-kDa collagen species in the sternal cultures were converted to lower molecular weight polypeptides.

Analysis of Procollagen mRNA Expression—To compare the observed differences in the synthesis of type I and II procollagen and types IX and X collagen in sternal and vertebral chondrocytes (Fig. 1) with differences in the levels of the mRNAs encoding these proteins, three types of analyses were carried out. A qualitative assessment of type I and II procollagen mRNAs and of type IX and X collagen mRNAs was carried out by Northern blot analysis of polyadenylated and total RNA, respectively, isolated from either vertebral or from sternal cells. The quantitative determination of each of these RNAs was then obtained from slot blot analyses of total RNA, while the number of copies of type I procollagen mRNAs/cell was obtained both from slot blot analysis and mRNA protection experiments.

Both sternal and vertebral chondrocytes expressed high levels of type II procollagen mRNAs but only vertebral cells expressed type I mRNAs detectable by Northern blots (Fig. 3). The α1(IX) mRNA was readily detectable on blots of sternal RNA but only visible in blots of vertebral RNA if longer autoradiographic exposures of blots containing two or three times more RNA were used, while type X collagen mRNA was detectable in vertebral but not in sternal chondrocyte RNA (Fig. 4) and remained undetectable even at higher RNA concentrations. Quantitative slot blot analyses of comparable mRNA quantities demonstrated that the type II procollagen mRNA level was 3.5 times greater in sternal cells than in vertebral cells. In contrast, type I mRNAs were undetectable in sternal cells while the level of type II mRNA was almost four times that of type I mRNAs in vertebral cells.

Because mRNA protection assays are about 10–50 times more sensitive than Northern blot analyses, the former were carried out to better assess the presence of type I procollagen mRNAs in sternal chondrocytes. The results are shown in Fig. 5, A–C and are summarized in Table II. Although only visible on the longer exposure of the autoradiograph (Fig. 5C), low levels of both proα1(I) and proα2(I) mRNAs could be detected in sternal cells. Since the mRNA copy number/cell
had been determined for proα1(I) and proα2(I) mRNAs in myoblasts, as described under "Materials and Methods," this analysis allowed an accurate and very sensitive determination of type I mRNA levels in both vertebral and sternal chondrocytes as well as in tendon fibroblasts and osteoblasts (Table II). Type I mRNA levels in sternal cells were 15-fold lower than in vertebral cells, over 100-fold lower than in osteoblasts. In contrast, the 8,000 copies/cell of type II mRNA in sternal cells was almost 3.5-fold higher than the 2,300 copies/cell in vertebral cells and 1.5 times greater than that of type I (proα1 and proα2) mRNAs in osteoblasts. While the difference between the type II procollagen mRNA levels in these two cell types might seem surprising, it is consistent with the finding that sternal cells synthesize twice as much collagen as vertebral cells, as well as the earlier results of Kravis and Upholt (1985) who reported that there were about 10,000 copies of type II mRNA/cell in RNA isolated from 17-day embryonic sterna, and about 2,000 copies/cell in RNA isolated from 7-day cultures of stage 24 limb mesenchyme.

The difference between the 4:1 ratio of type II to type I procollagen mRNAs levels in vertebral cells, and the 7:1 ratio of type II to type I α chain synthesis in these cells, determined from the data in Fig. 1A, suggests that type II procollagen mRNAs were more efficiently translated in vertebral cells than type I mRNAs, but only by a factor of less than two. If pepsin digestion had preferentially digested the α2(I) collagen, the levels of type I collagen synthesis may have been underestimated, and there may be no difference in the translation efficiency of these mRNAs. However, if one compares the 8-fold higher levels of type I procollagen mRNA in osteoblasts to that in vertebral chondrocyte with the 50-fold higher α chain synthesis in these two cell types, obtained from the data in Fig. 1B, it is apparent that type I procollagen mRNAs were about six times more efficiently translated in osteoblasts than in vertebral chondrocytes.

**Analysis of 5' Ends of Type I Procollagen mRNAs—** The apparent inefficient translation of type I mRNAs in vertebral chondrocytes could have resulted from a chondrocyte-specific factor which is limiting for collagen translation in vertebral cells, or it could result from an altered primary structure of the mRNA, such as the loss of the poly(A) tail or altered 5' ends of the type I mRNAs. However, Northern blot analyses of oligo(dT)-bound vertebral RNA (Fig. 3) showed that both proα1(I) and proα2(I) collagen mRNAs were polyadenylated, and mRNA protection experiments using *in vitro* synthesized transcripts containing the 5' ends of both type I mRNAs (Fig. 5, A and B) clearly demonstrated that the 5' ends of both of these mRNAs were the same in vertebral chondrocytes as in osteoblasts, tendon fibroblasts, and myoblasts in which they were efficiently translated. In order to independently verify the 5' sequence of α1(I) and α2(I) mRNAs found in vertebral cells, primer extension analysis was carried out. Because an alternate splicing pattern might occur for exon two of the proα2(I) gene (Aho et al., 1984; Tate et al., 1983), a primer was constructed corresponding to the first 15 base pairs of exon three (Tate et al., 1983). This should have resulted in a fully extended product 229 bases long. Since no similar alternate splicing could be seen in the DNA sequence of the first four exons of the proα1(I) gene (Finer et al., 1987), a 14-base pair primer ending in the Accl site at +93, shown in Fig. 5A, was used. As can be seen from these results (Fig. 6), both primers resulted in extensions having the predicted size for calvaria and vertebral mRNAs. These results confirm the RNA protection data and show that the 5' ends of both type I mRNAs are identical in RNA isolated from embryonic calvaria and vertebral chondrocytes.

**In Vitro Translation of RNA Isolated from Vertebral Cells—** Since other secondary sequence modification may affect mRNA translation ability but not be detected by primer extension or mRNA protection assays, the *in vitro* translatability of type I and II mRNAs was tested in a reticulocyte lysate translation system using increasing concentrations of polyadenylated RNA from vertebral cells. The fluorograph of the gel showing the [3H]leucine labeled proteins obtained is shown in Fig. 7. At the lowest RNA concentration, type II procollagen mRNA was more efficiently translated than either type I procollagen mRNA. Even though the proα1(I) and proα2(I) bands were expected to be less intense than the proα1(II) band since there were four times more type II than type I mRNAs in the vertebral RNA sample, these bands are barely visible. At the three higher RNA concentrations, however, the type I procollagen mRNAs were much more efficiently translated than type II mRNAs, with no proα1(II)
Fig. 5. Analysis of type I collagen 5' ends by RNA/RNA hybridization and RNase protection.
Molecular weight markers (M) are shown in each panel; cell types from which total RNA was derived and amounts of mRNA added are denoted in the figure. tRNA control (C) and probe (P) by itself are shown in A and B, respectively. Panel A: (top), proα1(I) protections; (bottom), map of the 5' end of proα1(I) gene. The thin line denotes 5'-flanking and intron sequence, and the thick line denotes exon sequence. Size of the protected fragment is given in the figure.
Panel B: (top), proα2(I) protections; (bottom), map of 5' end of proα2(I) gene. Panel C: 48-h exposure of the lanes containing sternal and vertebral RNA protections of proα1(I) and proα2(I) 5' ends to demonstrate the presence and relative concentrations of type I mRNAs in sternal and vertebral mRNA samples.

band visible at the two highest RNA concentrations. This unexpected effect of RNA concentration on the relative translation efficiencies of type I and II procollagen mRNAs appears to be unique to vertebral RNA. When mRNA from sternal cell was translated together with increasing amounts of mRNA from calvaria, there was no decrease in the intensity of the proα1(II) in comparison to the proα1(I) band at the higher RNA concentrations. This suggests that there may have been an inhibitor of type II mRNA translation in the vertebral RNA preparation. Such an inhibitory factor has been identified in mRNA isolated from a chondrosarcoma tumor (Paglia et al., 1981). Whatever the cause of the odd dependence of the translation efficiency on RNA concentration, the type I procollagen mRNAs are clearly capable of being translated into full length type I prepropeptides in vitro at moderate to high RNA concentrations.

DISCUSSION

Chondrocytes which undergo endochondral bone replacement and chondrocytes which remain as hyaline cartilage have previously been found to differ in that short chain type
myoblast values using the following equation:

\[
\text{Number of type I mRNA molecules/cell} = \frac{\text{densitometric area} \times \text{RNA/DNA ratio}}{\text{copy number of a myoblast}}
\]

These values were independently confirmed by slot or dot blot analysis. Myoblast copy number was determined using calculations presented under “Materials and Methods.” Number of type II mRNA molecules/cell were determined from slot blot analysis and back hybridization of the probe to itself as described under “Materials and Methods.” All values were determined from averages of at least two duplicate experimental determinations, and variations in calculated copy number were ±12%.

| mRNA | \(a1(II)\) | \((a1 + a2)(I)\) | \(a1/a2\) ratio |
|------|------------|-----------------|-----------------|
| Osteoblast | ND* | 5100 | 2.2 |
| Myoblast | ND | 2000 | 1.9 |
| Tendon fibroblast | ND | 3400 | 2.1 |
| Vertebral chondrocyte | 2800 | 630 | 1.6 |
| Sternal chondrocyte | 8000 | 40 | 3.0 |

* ND, not determined.

X collagen is only expressed by the former while another short chain collagen, type IX, is primarily expressed by the latter (Reginato et al., 1986; Jimenez et al., 1985; Ninomiya et al., 1986; Gibson et al., 1984; Schmid and Linsenmayer, 1985; Habuchi et al., 1985; Gibson and Flint, 1985; Capasso et al., 1984; Schmid and Linsenmayer, 1983, 1985; Gibson et al., 1982). While confirming this differential expression of the two short chain collagens, we have also shown that these two cell types differ in that vertebral chondrocytes, which undergo endochondral replacement, express moderate levels of both type I procollagen mRNAs which are translated into type I procollagen chains both in vivo and in vitro, while caudal sternal chondrocytes, which remain as hyaline cartilage, do not. Moreover, sternal cells expressed twice as much collagen as vertebral cells, but type II procollagen was more efficiently processed to \(\alpha\)-chains in vertebral chondrocytes than in sternal cells.

By making a quantitative determination of the relative rates of type II and type I procollagen synthesis in embryonic chick vertebral and sternal chondrocytes, the rates of type I chain synthesis in vertebral cells and osteoblasts, and of the number of mRNA molecules/cell in each of these cells as well as in myoblasts and tendon fibroblasts, we were able to show that type I procollagen mRNAs are about as efficiently translated in vertebral chondrocytes as type II mRNA, but only about a sixth as efficiently translated in vertebral chondrocytes as in osteoblasts. This lower translational efficiency was shown not to result from an altered structure of the type I mRNAs in vertebral cells, but type I1 procollagen was more efficiently translated in vertebral chondrocytes than in sternal cells.

Fig. 7. In vitro translation of type I and II procollagen poly(A)+ mRNA isolated from vertebral cells; relative efficiency of translation as a function of RNA concentration. Amount of RNA added/25 \(\mu\)l and positions of \(\alpha\)-chains are denoted in the figure. In vitro translation products were analyzed on a 5–10% SDS-polyacrylamide gel. C denotes translation of calvaria mRNA which was used as a control.
results in the preferential translation of type II relative to type I procollagen mRNA. The other which is active at moderate to high vertebral RNA concentrations clearly inhibits type II but not type I procollagen mRNA translation in vitro. Our results confirm earlier reports that embryonic vertebral chick chondrocytes contain moderately high levels of both type II and type I procollagen mRNAs but differ from reports that type I mRNAs were not translated either in vivo or in a reticulocyte lysate (Focht and Adams, 1984).

Our results also differ from reports of moderate levels of type I procollagen mRNAs in chondrocytes isolated from either whole 14-day embryonic chick sternum (Saxe et al., 1985), or from 7-day embryonic chick limbs (Kosher et al., 1986), which are not translated into detectable amounts of type I procollagen mRNA. The other which is active at vertebral chick chondrocytes contain moderately high levels of its type I but not type I procollagen mRNA translation in these cells, or in vitro (Pawlowski et al., 1981). It is important to note that in two cases in which translational regulation has been reported, and the ratio of proα1 to proα2 mRNA determined, the suppression of type I collagen expression coincides with a large excess of proα2(I) relative to proα1(I) mRNA in the cytoplasm, with proα(I)/proα2(I) ratios as low as 0.3 and 0.4 (Saxe et al., 1985; Kosher et al., 1986) rather than the value of 2.0 ± 0.2 found in type I expressing tissues, shown in Table II and reported previously (deWet et al., 1985), or the value of 1.6 found for vertebral chondrocytes (Table II). Based on the available data, the expression of mature proα1(I) collagen mRNA in chondrocytes appears to be directly or indirectly regulated by the physical state of the cells, with very little if any expression in cells in suspension (Finer et al., 1985; Saxe et al., 1985; Kosher et al., 1986), but moderate levels of expression in adherent cells, such as the embryonic chick vertebral cells in this report, or very similar adherent vertebral cells studied by Alema et al. (1985), in which type I collagen synthesis was readily detected.

While we cannot completely rule out the possibility that type I collagen expression in adherent chondrocytes is due to fibroblast contamination, a rigorous cell selection was used to select only those cells which remained in suspension for seven days, the ratio of proα1(I) to proα2(I) mRNA of 1.6 is significantly lower than the value of 2.0 ± 0.2 in fibroblast RNA preparations, and the fact that type I procollagen mRNAs are six times more efficiently translated in osteoblasts than in vertebral cells (Fig. 1B and Table II), all support the conclusion that the type I mRNAs and protein we have identified in vertebral chondrocytes appears to be directly regulated by the type I1 but not type I procollagen mRNAs in chondrocytes characterized here were prepared from the caudal half of the phalic half, a tissue which undergoes endochondral bone replacement, expresses type X collagen (Ninomiya et al., 1986; Reginato et al., 1986; Gibson and Flint, 1985; Gibson et al., 1984), and evidently type I procollagen mRNAs as well, although with an excess of proα2(I) mRNA and a concomitant absence of detectable amounts of type II collagen.

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REFERENCES

Adams, S. L., Boettiger, D., Focht, R. J., Holscher, H., and Pacifici, M. (1982) Cell 30, 373–384
Abo, S., Tate, V., and Boedtker, H. (1984) Nucleic Acids Res. 12, 6117–6125
Alema, S., Tato, F., and Boettiger, D. (1985) Mol. Cell. Biol. 5, 558–544
Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88
Bornstein, P., and Sage, H. (1980) Annu. Rev. Biochem. 49, 957–1004
Capasso, O., Tajana, G., and Cancedda, R. (1984) Mol. Cell. Biol. 4, 1163–1168
deWet, W. J., Chu, M., and Prockop, D. J. (1983) J. Biol. Chem. 258, 14385–14389
Finer, M. H., Abo, S., Gerstenfeld, L. C., Boedtker, H., and Doty, P. (1987a) J. Biol. Chem. 262, 13323–13332
Finer, M. H., Boedtker, H., and Doty, P. (1987b) Gene 56, 71–78
Finer, M. H., Gerstenfeld, L. C., Young, D., Doty, P., and Boedtker, H. (1988) Mol. Cell. Biol. 5, 1415–1424
Focht, R. N., and Adams, S. L. (1984) Mol. Cell. Biol. 4, 1843–1852
Fraenkel, A. M., Garoff, H., and Lelahch, J. (1986) Nucleic Acid Res. 8, 4541–4549
Gerstenfeld, L. C., Chipman, S. D., Glowacki, J., and Lian, J. (1987) Dev. Biol. 122, 49–60
Gerstenfeld, L. C., Finer, M. F., and Boedtker, H. (1985) Mol. Cell. Biol. 5, 1425–1433
Gerstenfeld, L. C., Beldekas, J. C., Franzblau, C., and Sonnenschein, G. E. (1985) J. Biol. Chem. 258, 12058–12063
Gerstenfeld, L. C., Crawford, D. R., Boedtker, H., and Doty, P. (1984) Mol. Cell. Biol. 4, 1483–1492
Gibson, G. J., Beaumont, B. W., and Flint, M. H. (1984) J. Cell Biol. 99, 208–216
Gibson, G. J., and Flint, M. H. (1985) J. Cell Biol. 101, 277–284
Gibson, G. J., Schor, S. L., and Grant, M. E. (1982) J. Cell Biol. 93, 767–774
Habuchi, H., Conrad, H. E., and Glasser, J. H. (1985) J. Biol. Chem. 260, 13029–13034
Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
Jimenez, S. A., Yankowski, R., and Regnato, A. M. (1986) Biochem. J. 233, 357–367
Kosher, H. A., Lyuky, W. M., and Gay, S. W. (1985) J. Cell Biol. 102, 1151–1156
Laemmli, U. K. (1970) Nature 227, 680–685
Laskey, R. A., and Mills, A. D. (1975) Eur. J. Biochem. 56, 335–341
Lehrach, H., Diamond, D., Woznay, J., and Boedtker, H. (1977) Biochemistry 16, 4743–4751
Lehrach, H., Frischau, A. M., Hanahan, D., Woznay, J., Fuller, F., Crenkmajovak, R., Boedtker, H., and Doty, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5417–5421
Lehrach, H., Frischau, A. M., Hanahan, D., Woznay, J., Fuller, F., and Boedtker, H. (1979) Biochemistry 18, 3145–3152
Linsenmayer, T., Gibney, E., and Little, C. (1982) Exp. Eye Res. 34, 371–379
Lish, H. F., and Nathan, D. G. (1972) J. Biol. Chem. 247, 7822–7829
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Martin, J., Gottesman, G. D., and Zaslouf, M. A. (1988) Biochemistry 27, 3351–3356
Maxam, A., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
Mayne, R., Hertod, B. W., Mayne, P. M., Sanderson, R. D., and Linsenmayer, T. (1984) Exp. Cell Res. 151, 171–182
Mayne, R., and Irwin, J. H. (1986) in Articular Cartilage Biochemistry (Kuetzmann, K., ed) pp 25–35, Raven Press, New York
Melton, D. A., Krieg, P. A., Rebagliti, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acid Res. 12, 7035–7056
Mirsky, A. E., and Ris, N. (1950) J. Gen. Physiol. 34, 451–462
Ninomiya, Y., Gordon, M., van der Rest, M., Schmid, T., Linsenmayer, T., and Olson, B. R. (1986) J. Biol. Chem. 261, 5041–5050
Ninomiya, Y., and Olsen, B. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3014–3018
Paglia, L. M., Wiestner, M., Duchene, M., Ouellette, L. A., Horlein, D., Martin, G. R., and Muller, P. L. (1981) Biochemistry 20, 3523–3527

Pawlowski, P. J., Brierley, G. T., and Lukens, L. N. (1981) J. Biol. Chem. 256, 7685–7696

Pelham, H. R. B., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247–256

Peterkofsky, B., and Diegelmann, R. F. (1971) Biochemistry 10, 988–994

Reginato, A. M., Lash, J. W., and Jimenez, S. A. (1986) J. Biol. Chem. 261, 2897–2904

Sax, S. A., Lukens, L. N., and Pawlowski, P. J. (1985) J. Biol. Chem. 260, 3812–3819

Schmid, T. M., and Conrad, H. E. (1982) J. Biol. Chem. 257, 12451–12457

Schmid, T. M., and Linsenmayer, T. F. (1983) J. Biol. Chem. 258, 9504–9509

Schmid, T. M., and Linsenmayer, T. F. (1985) J. Cell Biol. 100, 598–605

Schwarz, R., Colarusso, L., and Doty, P. (1976) Exp. Cell Res. 102, 63–71

Sonenshein, G. E., Sieklvitz, M., Siebert, G. R., and Gefter, M. L. (1978) J. Exp. Med. 148, 301–310

Tate, V., Finer, M., Boedtker, H., and Doty, P. (1983) Nucleic Acids Res. 11, 91–104

Thomas, P. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201–5205

van der Rest, M., Mayne, R., Ninomiya, Y., Seidah, N. G., Cretien, M., and Olsen, B. R. (1985) J. Biol. Chem. 260, 220–225

van der Mark, K., van Menkel, M., and Wiedemann, H. (1984) Eur. J. Biochem. 138, 629–633

Wu, J., and Eyre, D. (1984) Biochem. Biophys. Res. Commun. 123, 1033–1039

Young, M. F., Vogeli, G., Nunez, A. M., Fernandez, M. P., Sullivan, M., and Sobel, M. E. (1984) Nucleic Acid Res. 12, 4207–4208