Gene Expression in Mineralizing Chick Epiphyseal Cartilage*

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The endochondral growth cartilage serves as an excellent model for study of the biological mineralization process. Since it exhibits both spatial and temporal separation of development events, it is possible to relate chondrocyte proliferation and hypertrophy to cartilage maturation and bone formation. Analyses of regions from the growth cartilage therefore provide a means for examining developmental mechanisms that direct formation of calcified cartilage and endochondral bone. In our previous studies of the calcification process, we have used this system to examine key metabolic events associated with cartilage and bone mineralization. Results of these studies have shown that with the onset of mineralization there are profound changes in the cell redox status and the energy charge ratio (1–3). The relationship between these metabolic changes and expression of mineralization specific proteins have not been explored.

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Of the considerable number of proteins present in the calcifying epiphysis, only a few have been directly linked with the mineralization process (4, 5). These include noncollagenous matrix proteins such as osteonectin and chondrocalcin and alkaline phosphatase (6–9). Recently, a low molecular weight species of collagen, type X, has been found in hypertrophic chondrocytes (10, 11). Analysis of the distribution of this collagen by immunocytochemical and protein isolation techniques has shown a clear relationship between localization of type X and the initiation of mineralization (10, 12–16).

Another approach to identifying proteins implicated in the mineralization process is to examine the appearance of selected mRNAs in calcifying tissues. However, due to the difficulty of isolating mRNA from nonembryonic cartilage and bone, analyses of mRNA levels in mineralizing tissues have generally been limited to studies using cultured chondrocytes or developing embryos (17–20). An alternative approach to identifying mRNAs in mineralizing systems has been in situ hybridization (21, 22). However, while this technique can provide information at the cellular level, the results are more qualitative than quantitative. We have addressed this analytical problem and report here procedures for isolating mRNA from chick epiphyseal cartilage and endochondral bone. Using these procedures, we have examined levels of mRNA for several collagen species and calcium-binding proteins in both whole epiphyseal cartilage and selected regions of the growth cartilage. Analyses of type I, type II, and type X collagen mRNA steady state levels indicate that type X collagen mRNAs are high not only in hypertrophic calcifying cartilage, but also in endochondral bone. Similar analyses indicate that osteonectin mRNA is present in nonmineralizing as well mineralizing regions of the cartilage, suggesting that its role in developing bone is broader than has hitherto been proposed.

EXPRESSIMENTAL PROCEDURES

Tissues—Epiphyseal cartilage and endochondral bone were obtained from the tibiae of 8-week-old chicks. The birds were killed by cervical dislocation, and the proximal tibial growth cartilage was exposed. Thin sections of the tissue were rapidly removed and frozen in liquid nitrogen or in guanidinium isothiocyanate. Two types of epiphyseal cartilage samples were prepared: 1) whole growth cartilage which included resting, proliferating, hypertrophic, and calcifying cartilage; 2) upper (precalcified) and lower (calcifying) growth cartilage regions. In the latter case, thin sections of tissue were removed from the resting/proliferating regions and pooled; similarly, sections from the hypertrophic and calcifying cartilage zones were collected and pooled. Identification of each zone was based on microscopic evaluation of transverse sections of the growth cartilage. While thickness of the growth cartilages varied from animal to animal, five to six chickens were usually sufficient to obtain 200–300 μg of RNA from each epiphyseal region. Following removal of the growth cartilage, slices of endochondral bone were removed from the metaphyseal region of the tibia using a scalpel. One mg of RNA could be obtained from the endochondral bone of two to three chicks.
Embryonic sternum and calvaria were obtained from 15-day chick embryos. Sterilized cartilage from 40 embryos, dissected free of soft tissues, yielded about 260 μg of RNA. Calvarial tissue from 40 embryos yielded approximately 1 mg of RNA.

**RNA Preparation**—All solutions and glassware used for RNA preparation were sterile, and all solutions were prepared using diethyl pyrocarbonate-treated double-distilled water. Tissue fragments were homogenized in 5 ml of 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM EDTA in a solution of 5 M guanidinium isothiocyanate with 5 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 M mercaptoethanol. Samples were centrifuged for 10 min at 6,000 rpm in a Sorvall SS 34 rotor, and the supernatants were combined with 0.4 g/ml chloroform and layered on top of 5.7 M CsCl containing 0.1 M EDTA (pH 7.5). After centrifuging overnight at 36,000 rpm (at 20°C) in an SW50.1 rotor, the resulting RNA precipitate was resuspended in 1-2 ml of 7.5 M guanidine HC1 and heated at 68°C for 2 min. The pH was adjusted to 5.0 with 1 M acetic acid, 0.5 volume of ethanol was added, and the large molecular weight RNA precipitated overnight at -20°C.

The precipitate was suspended in 1 ml of buffer containing 0.3 M sodium acetate (pH 6.0), 10 mM EDTA, and 0.5% SDS and heated to 60°C. One ml of sodium acetate-saturated phenol (pH 6.0) was added to the suspension, and the mixture was shaken at 60°C for 10 min with 1.2 ml of chloroform-isoamyl alcohol (24:1). After further shaking for 15 min at 60°C, the mixture was centrifuged briefly at 4°C. The aqueous layer was removed, re-extracted at least twice at 60°C with phenol/chloroform, and then twice at room temperature with chloroform. MgCl2 was added to a final concentration of 10 mM, and the RNA was precipitated overnight with 2.5 volumes of ethanol. The resulting pellet was washed with 70% ethanol and resuspended in diethyl pyrocarbonate-treated water. RNA concentrations were determined both by absorbance at 260 nm and by ethidium bromide spot tests (24). Samples were divided into aliquots and stored at -70°C as ethanol precipitates.

**Gene Electrophoresis of RNA**—RNA was examined after electrophoresis on 1% agarose gels and staining with ethidium bromide to ensure that each probe was hybridizing to mRNA species of the correct size. Quantitation of mRNA levels was carried out with dot blots containing three concentrations (100-150 ng) of each RNA prepared. Autoradiographs of the dot blots were analyzed by scanning with a video-digitizer image processor (25), and the data were expressed as the integrated optical density of each spot.

**Probes**—All of the probes were prepared from subclones placed in Promega Biotec riboprobe vectors in the appropriate orientation for producing radioactive RNA transcripts complementary to the mRNA.

The type I collagen RNA was an 816-bp EcoRI-TaqI fragment from the carboxypropeptide and 3' untranslated sequences of a chick genomic α2(I) collagen gene clone (25) subcloned into pGEM4 by Dr. Sherrill Adams (University of Pennsylvania). The type II collagen probe was derived from the 1.25-kb cDNA clone pCs-2 (26); we subcloned the 800-bp PvuII-HindIII fragment containing carboxypropeptide and 3'-untranslated sequences into pGEM4. Type X collagen was subcloned from the 0.6-kb cdNA insert of pYN3-116 (11); a 183-bp HindIII-BamHI sequence from the 5' end of the insert was cloned into pGEM3. The calmodulin probe contained a 277-bp EcoRI-PstI fragment in the coding region; this was subcloned into pGEM4 from the full length calmodulin cDNA clone pCB12 (17). The probe for γ-actin, isolated by Cleveland et al. (28), was a 470-bp HindIII insert from the 3' untranslated region cloned in pBR322.

All of the above probes are derived from chicken cDNA or genomic DNA sequences. The osteocalcin probe was a 0.9-kb EcoRI fragment from a bovine cDNA clone (29) which was subcloned into pGEM3. The bovine cDNA clone had previously been shown to cross-hybridize to chicken RNA (29).

**RESULTS**

**Preparations of RNA from Cartilage and Bone**—The isolation of cellular RNA from cartilage and bone is complicated by the presence of large quantities of negatively charged proteoglycans which co-purify with nucleic acids. For this reason, published studies of connective tissue mRNA have utilized nucleic acids derived from either primary cells in culture or, less frequently, sternum and calvaria from embryos. Since an essential feature of the present study is analysis of mRNA levels from specific regions of tibial epiphyseal cartilage and bone, we have explored a variety of methods for preparation of hybridizable RNA from these tissues.

The most commonly used procedure for isolating RNA from tissues involves centrifugation of a guanidinium isothiocyanate tissue homogenate through a 5 M cesium chloride cushion (30). When chick epiphyseal cartilage or endochondral bone homogenates were subjected to this procedure, the resuspended pellet was viscous and the majority of ethidium bromide-staining material remained in the well of a 1% agarose gel (Fig. 1, lane B). Similar results were obtained when homogenates in 4 M guanidinium isothiocyanate were extracted with hot phenol (25) and or precipitated in 7.5 M guanidinium HCl with 0.5 volume of ethanol (30, 31). However, a combination of cesium chloride density centrifugation and guanidinium HCl-ethanol precipitation followed by hot phenol extraction resulted in a nonviscous RNA solution which showed a classical pattern of large and small ribosomal RNAs along with diffuse mRNA on agarose gels (Fig. 1, lane A).

The quality of mRNA prepared by this method was examined by electrophoresis on agarose-formaldehyde gels, Northern blotting onto nylon membranes, and hybridization to cloned gene probes. Results with RNA from endochondral bone 3- to 5-week chickens are shown in Fig. 2. Collagen probes indicated α1(type I) collagen mRNA of approximately 4.6 and 4.8 kb and type X mRNA of 2.4 kb. A major band at 1.6 kb and a faint band at 4 kb were observed for calmodulin mRNA, while the bovine osteocalcin probe hybridized to a major band at 1.8 kb and a minor RNA band at 2.2 kb. Northern blots of epiphyseal cartilage RNA hybridized to the probe for α1(type II) collagen yielded a single band at 5.2 kb (data not shown). These RNA preparations therefore con-
embryo sterna and calvaria. RNA from at least three different types of tissue homogenate in 5 M guanidinium isothiocyanate followed by centrifugation of tissue homogenate in 5 M guanidinium isothiocyanate followed by guanidine HCl precipitation and hot phenol extraction. B, same preparations prior to hot phenol extraction. C, λ DNA HindIII and X174 RF DNA HincII size markers (Pharmacia LKB Biotechnology Inc.).

Relative Levels of mRNAs—Tissue-specific differences in mRNA levels were assessed by dot-blot hybridizations in which RNA from epiphyseal cartilage and endochondral bone of 8-week chicks were compared with RNA from 15-day chick embryo sterna and calvaria. RNA from at least three different preparations of each tissue were spotted on filters and hybridized to probes. Results of one series of hybridizations with collagen type I, II, and X, as well as osteonectin, are shown in Fig. 3. In order to confirm that quantitation of RNA by A260 and ethidium bromide assays accurately represented levels of mRNA in the preparations, γ-actin mRNA of several tissues was also measured. Autoradiographs were analyzed by densitometric scanning. The tissue with the highest optical density reading was assigned an abundance value of 1.0; abundance values for other tissues on the filter hybridized to the same probe were calculated in relationship to this value (Table I).

As expected, RNA from embryonic sternum contained high levels of type II collagen mRNA with no detectable type I, whereas calvarial RNA contained type I collagen mRNA but no type II mRNA. Epiphyseal cartilage, like sternum, had type II but not type I mRNA; however, the level of type II RNA/µg of RNA was lower than that seen in sternum. Endochondral bone showed low levels of type II mRNA and significant levels of type I mRNA; however, type I mRNA levels in this tissue never approached those seen with calvaria. More surprising results were obtained when dot blots were hybridized to osteonectin and type X collagen probes. Message for osteonectin, originally described as a bone-specific protein (6), was seen in all tissues examined. Levels of osteonectin mRNA in a given tissue were considerably more variable than those of other mRNAs. Type X collagen is reported to be a product of hypertrophic chondrocytes (10-18); highest levels of type X mRNA would therefore be expected in the hypertrophic cartilage region of epiphyseal cartilage. While epiphyseal cartilage contained type X message, endochondral bone consistently showed much higher levels of this mRNA.

Levels of type II and X collagen mRNAs in various regions of endochondral bone and epiphyseal cartilage were determined by Northern blot analysis. RNA preparations of each tissue were spotted in triplicate on nylon membranes and hybridized to 32P-labeled riboprobes. The first three panels show results with 5 µg of RNA; the lane hybridized to calmodulin contained 10 µg of RNA. Exposure time for each gel corresponded to the optical density reading assigned to each tissue. Autoradiographs of two blots which had been hybridized to different probes with potentially different specific activities. The relative levels of type II and X, as well as osteonectin, are shown in Fig. 4.

Relative hybridization results are shown in Table I. Hyridization analyses indicated that the RC-PC region of epiphyseal cartilage contained high levels of type II collagen mRNA and little or no type X mRNA; in contrast, the HTC-CC region showed lower levels of type II mRNA and significant amounts of type X message (Fig. 6). Neither of these RNA preparations contained type I collagen mRNA. Comparison of RNA from the HTC-CC region with that from endochondral bone indicated that type X mRNA levels in bone were 67% of those seen in the adjacent HTC-CC region, while type II levels in bone averaged 22% of those in the HTC-CC region. Although whole epiphyseal cartilage showed levels of type II mRNA approximately one-half those in sternum, the amount of type II mRNA in the RC-PC zone was equivalent to that seen in 15-day embryo sternum.

To directly compare the ratios of different collagen mRNAs within a given tissue, dot blots were prepared in which no insert were spotted along with RNA from the HTC-CC region and endochondral bone. These blots were hybridized to nick-translated plasmids containing type I, II, and X collagen sequences. Hybridization of 32P-labeled plasmid sequences to the plasmid DNA on each blot provided a means of comparing autoradiographs of two blots which had been hybridized to different probes with potentially different specific activities. The relative levels of type II and X, as well as osteonectin, are shown in Fig. 4.
**Fig. 3. Dot-blot hybridizations to chick tissues.** Increasing amounts of RNA were hybridized to riboprobes for type I (A), type II (B), type X (C), and osteonectin (D). Tissues: E, epiphyseal cartilage; S, sternum; C, calvaria; B, endochondral bone.

**TABLE I**

Relative abundance of mRNA in chick tissues

The tissue with the highest optical density for a given probe is assigned the value 1.0; other values with the same probe are calculated relative to 1.0. Thus, numerical comparisons are only valid within a given row. Sternum and calvaria RNA were from 15-day chick embryos; RNAs from whole epiphyseal cartilage and endochondral bone were isolated from 8-week chicks. Average values are derived from hybridization of probe to at least three different preparations of each tissue.

| Probe         | Tissue          | Sternum | Epiphyseal cartilage | Endochondral bone | Calvaria |
|---------------|-----------------|---------|----------------------|-------------------|----------|
| Collagens     |                 |         |                      |                   |          |
| Type I        |                 | 0⁴      | 0.30 (0.11-0.49)      | 1.0               |          |
| Type II       |                 | 1.0     | 0.47 (0.42-0.53)      | 0.10 (0.02-0.24)  | 0        |
| Type X        |                 | 0       | 0.20 (0.12-0.32)      | 1.0               | 0        |
| Osteonectin   |                 | 0.46 (0.12-0.62) | 0.06 (0.03-0.11) | 0.80 (0.49-1.7)  | 1.0      |
| Calmodulin    |                 | 1.0     | 0.45 (0.32-0.59)      | 9.56 (0.41-0.71)  | 0.52     |
| Actin         |                 | 1.14 (0.8-1.47) | 1.0                  | 0.85 (0.31-0.80) | (0.67-1.03) |

⁴ Ratio to tissue of highest abundance = 1.00.
⁵ 0, not detected after 24-h film exposure with two intensifying screens.
⁶ Numbers in parentheses equal range of values.
⁷ -, not determined.

Results of these hybridizations indicated that in the HTC-CC zone the type II levels were higher than type X mRNA, whereas there was almost 5 times as much type X as type II mRNA in endochondral bone (Table II). While type X mRNA levels in the bone were relatively high, levels of type I mRNA were 2.5 times higher.

Analyses of osteonectin mRNA levels in the two regions of epiphyseal cartilage indicated that presence of this message was not correlated with calcification of the tissue; indeed, the resting/proliferating region showed greater amounts of osteonectin mRNA than did the hypertrophic/calcified cartilage region (Fig. 6).

**Calmodulin mRNA Levels**—RNA preparations from chick embryo calvaria and sternum and 8-week chicken endochondral bone were compared with RNA from chicken liver as well as brain. These tissues were selected for comparison because brain contains high levels of calmodulin whereas liver has relatively low levels of this protein (32). Dot blots of these RNAs hybridized to a ³²P-riboprobe of chick calmodulin are presented in Fig. 7. The levels of calmodulin mRNA in developing calvaria and bone were low and similar to those in noncalcifying embryo sternum; liver and brain calmodulin mRNA levels were 3-fold and 20-fold higher, respectively.

**DISCUSSION**

A double extraction procedure for preparing RNA has permitted us to carry out a quantitative analysis of mRNA changes associated with cartilage maturation and bone formation in chick tibia. Using this technique, we have correlated...
levels of mRNA coding for types I, II, and X collagens and the calcium-binding proteins calmodulin and osteonectin with stages of chondrocyte maturation in growth cartilage.

The observation that type X collagen mRNA is found in the HTC-CC region but not in the RC-PC region is consistent with immunolocalization studies of type X protein and lends strong support to the idea that this protein is associated with the mineralization of cartilage (12, 13, 16, 33, 34). Further support for the role of type X collagen in biological mineralization is our finding that endochondral bone contains high levels of type X collagen mRNA. At this stage, it is not possible to state how type X collagen may regulate mineral deposition. However, the observation that this collagen was associated with matrix vesicles (14) suggests that it may form a permissive environment for apatite development.

While the relationship between initiation of type X collagen synthesis and chondrocyte maturation has been extensively studied, there is less information available on the maintenance of type X levels during bone formation. Immunolocalization studies of chick tibiotarsus (10, 13, 33) suggested that type X collagen is present not only in calcified cartilage but also in bone. Results of our study indicate significant steady state levels of type X mRNA in endochondral bone. Thus, it is probable that the protein found in this region is synthesized by cells present in the bone.

Since trabeculae of calcified cartilage are frequently seen within the diaphysis, we examined the possibility that type X mRNA in bone preparations was a product of hypertrophic chondrocytes. Using type II mRNA as an index of chondrocyte activity, we examined the ratio of type X/type II mRNA in regions of the epiphysis. Endochondral bone preparations showed a ratio 7 times greater than that in the hypertrophic calcified cartilage region. One possible explanation for the high ratio in bone is that type X mRNA in endochondral bone is produced by a subset of hypertrophic chondrocytes which persist into the diaphysis and which differ from the average chondrocyte found in the HTC-CC zone in that they synthesize predominantly type X collagen mRNA. Alternatively, there may exist bone cells which are capable of synthesizing type X mRNA. Indeed, we have occasionally obtained...
endochondral bone RNA preparations containing high levels of type X mRNA with little type I and no type II mRNA. Biochemical analyses of collagen types in developing chick sterns show that cells synthesizing predominantly type X are also seen during sternal calcification; Gibson and Flint (15) have reported that newly synthesized collagen in the cephalic portion of sponda from 18–19-day embryos is 80–90% type X. We propose that in the calcifying growth cartilage, there is a spatial localization of cells which produce type X as the major or sole collagen message. These cells may be present in late hypertrophic/calcifying cartilage and remain active during endochondral bone formation. We are currently examining this hypothesis using in situ hybridization techniques.

Although our results are consistent with the hypothesis that type X collagen is concerned with the mineralization of cartilage and bone, they are less clear with respect to osteonectin. This calcium-binding phosphoprotein is a major non-collagenous component of bone which had been postulated to play a key role in osteogenesis by initiating mineral deposition and by linking mineral to the collagenous matrix (6, 28). However, osteonectin has recently been reported to be identical with the SPARC protein which is a major secreted product of a variety of cell types involved in synthesis of basement membrane components or in steroid production (34, 35). Studies reported here indicate that, while high levels of osteonectin mRNA are indeed found in endochondral bone, significant amounts are also seen in premineralizing chick embryo sterns. Furthermore, osteonectin mRNA is present in higher amounts in the proliferating region of epiphyseal cartilage than in the hypertrophic and calcifying cartilage region. Preliminary results using antibody to chick osteonectin indicate that the high steady state levels of osteonectin mRNA in the region containing proliferating chondrocytes are paralleled by significantly high levels of the protein in this region (2, 3). These results demonstrate that osteonectin synthesis in chick tibia is not limited to regions of mineral formation.

The fact that osteonectin binds calcium with high affinity and delays hydroxyapatite-seeded crystal growth (36) has led to recent proposals that osteonectin/SPARC may serve as a buffer for calcium in cartilage and, during mineralization, may modulate crystal growth in the extracellular matrix (34, 35, 37, 38). If the osteonectin in bone functions to control matrix mineralization, then our results suggest either that it serves a different role in proliferating cartilage or that its synthesis in epiphyseal cartilage is activated prior to its requirement for the mineralization process.

Our studies of calmodulin mRNA were based on 1) the observation that cells in the HTC-CC zone contain high levels of calcium (39); and 2) immunofluorescence studies of rat skeletal tissues indicating high calmodulin levels in mature and mineralizing chondrocytes (40). The present studies show no correlation between calmodulin mRNA levels and the development of mineralization; furthermore, calmodulin mRNA levels are lower in all of the connective tissues examined than they are in either liver or brain. Thus, it is unlikely that calcification-related intracellular calcium flux is mediated by this protein. We are currently examining the possibility that other calcium-binding proteins such as osteonectin/SPARC or alkaline phosphatase may regulate calcium levels in mineralization tissues.

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