Collagen Gene Expression during Limb Cartilage Differentiation

Robert A. Kosher, William M. Kulyk, and Steven W. Gay
Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06032

Abstract. As limb mesenchymal cells differentiate into chondrocytes, they initiate the synthesis of type II collagen and cease synthesizing type I collagen. Changes in the cytoplasmic levels of type I and type II collagen mRNAs during the course of limb chondrogenesis in vivo and in vitro were examined using cloned cDNA probes. A striking increase in cytoplasmic type II collagen mRNA occurs coincident with the crucial condensation stage of chondrogenesis in vitro, in which prechondrogenic mesenchymal cells become closely juxtaposed before depositing a cartilage matrix. Thereafter, a continuous and progressive increase in the accumulation of cytoplasmic type II collagen mRNA occurs which parallels the progressive accumulation of cartilage matrix by cells. The onset of overt chondrogenesis, however, does not involve activation of the transcription of the type II collagen gene. Low levels of type II collagen mRNA are present in the cytoplasm of prechondrogenic mesenchymal cells at the earliest stages of limb development, well before the accumulation of detectable levels of type II collagen. Type I collagen gene expression during chondrogenesis is regulated, at least in part, at the translational level. Type I collagen mRNAs are present in the cytoplasm of differentiated chondrocytes, which have ceased synthesizing detectable amounts of type I collagen.

As a first step in studying the molecular regulation of the expression of type I and type II collagen genes during chondrogenesis, we have used cloned cDNA probes to examine changes in the cytoplasmic levels of type I and II collagen mRNAs during chick limb cartilage differentiation in vitro and in vivo. In particular, we have determined steady-state cytoplasmic collagen mRNA levels during the course of the chondrogenic differentiation of the distal subridge mesenchymal cells of stage 25 (12) chick wing buds in micromass culture (10). The micromass culture of stage 25 subridge mesenchymal cells is a system in which a relatively homogeneous population of undifferentiated chondrogenic progenitor cells uniformly progress through the phases of chondrogenesis unencumbered by the formation of the large amount of noncartilage tissue that differentiates in conventional cultures derived from the cells of whole limb buds (10). This investigation thus extends the recent study of Kravis and Upholt (22) on developmental changes in type II collagen mRNA levels in whole limb buds and in cultures prepared from whole limb buds. We have found, as expected, that the level of cytoplasmic type II collagen mRNA progressively increases during the course of chondrogenesis. An unexpected finding of considerable interest was the presence of low levels of type II collagen mRNA in the cytoplasm of prechondrogenic mesenchymal cells at the earliest stages of limb development, indicating the type II collagen gene is being transcribed well before the onset of overt cartilage differentiation and the accumulation of detectable amounts of type II collagen. Furthermore, our results are consistent with other recent studies (1, 9, 31) indicating that type I collagen gene expression during...
chondrogenesis is regulated, at least in part, at the translational level, since substantial amounts of type I collagen mRNAs are present in the cytoplasm of well-differentiated chondrocytes which do not synthesize detectable amounts of type I collagen.

**Materials and Methods**

**Preparation of Tissue and Cell Culture**

Wing buds were removed from stage 18/19 and stage 25 (12) embryos of White Leghorn chicks. Dorsal wing bud tips (subridge regions) were cut away from the stage 25 subridge bud as previously described. The size of the excised subridge regions being 0.3 - 0.4 mm from the distal apex of the tissue to the proximal cut edge (19). Ectoderm was removed from the stage 18/19 wing buds and stage 25 subridge regions, and micromass cultures were prepared from the distal subridge mesenchymal cells of the stage 25 wing buds as previously described (10). Purified cartilage rudiments were prepared from the limbs of 7-8 day embryos as described by Linzenmayer (24). Strips were dissected from 12-d embryos with special care being taken to remove perichondrial tissue. GH3 cells, a clonal line of rat pituitary tumor cells, were supplied by Dr. Bruce White (University of Connecticut Health Center). Total RNA purified from 17-d embryonic chick calvaria was supplied by Dr. William Upholt (University of Connecticut Health Center).

**Hybridization Probes**

Two cloned type II collagen cDNA plasmids were used: pCAR1 and pCAR2, 496-base pair (bp) and 680-bp cDNA inserts, respectively, into pBR322 (37). The type I collagen cDNA plasmids used were pCGh, a 2,500-bp cDNA insert into pBR322 encoding part of the α2(I) collagen subunit (23), and pCAL1, a 675-bp cDNA insert corresponding to α1(I) collagen mRNA (38). As a control probe we have used the cloned rat growth hormone cDNA plasmid, GH1-pBR322 (13). Plasmids pCAR1, pCAR2, and pCAL1 were supplied by Dr. William Upholt (University of Connecticut Health Center).

**Hybridization Analyses**

Cytoplasmic mRNA levels were measured in subridge limb mesenchymal cells at various times during micromass culture by the cytoplasmic dot hybridization procedure of White and Bancroft (40), except that 10 mM vanadylribonucleoside complex was included during preparation of cytoplasmic extracts, and aliquots of cell suspensions were removed for determination of total DNA content. Cytoplasmic extracts were prepared from intact limb mesoderm and cartilage by homogenizing the tissues in a Potter-Elvehjem homogenizer in ice-cold 10 mM Tris (pH 7), 1 mM EDTA containing 0.5% Nonidet P-40, and 10 mM vanadylribonucleoside complex. The homogenates were filtered through a 20-μm mesh Nitex monofilament screen, and aliquots were removed for DNA determination (4). The nuclei were pelleted by centrifugation, and the supernatants treated as described (40).

Aliquots of the cytoplasmic extracts were diluted with 15X SSC (standard saline citrate, 0.15 M NaCl/0.015 M sodium citrate) and spotted onto nitrocellulose using the Schleicher & Schuell micro-sample filtration manifold (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose dot blots were baked at 80°C in a vacuum oven for 90 min, and then prehybridized overnight at 42°C in a solution containing 50% formamide, 4X SSC, 50 mM sodium phosphate buffer (pH 6.5), 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.1% SDS, and 100 μg/mL of denatured salmon sperm DNA. Hybridization was then carried out for 48 h at 55°C in a solution identical to the above except for the addition of 0.1 g/mL of dextran sulfate and 1.5 x 10^5 cpn of the appropriate heat-denatured cloned cDNA plasmid. The nitrocellulose dot blots were washed four times for 5 min each at 55°C in 2X SSC, 0.1% SDS; four times for 15 min each at 55°C in 0.1X SSC, 0.1% SDS; and, exposed to Kodak X-OMAT XAR-5 film for various lengths of time at −70°C with a Dupont Cronex lightning plus intensifying screen. The levels of hybridizable RNA sequences were quantified by scanning the dots of the resultant autoradiograms with an SL-TRFF soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA), interfaced to an Apple Ile computer programmed to provide automatic peak integration. Several film exposures were scanned to be sure the densities of the dots were in the linear response range of the film.

In one series of experiments, a slight modification of the dot-blot hybridization procedure described by Cheley and Anderson (5) was used to measure specific RNA sequences. Briefly, limb tissue was solubilized by sonication in 7.6 M guanidine-HCl in 0.1 M potassium acetate buffer, pH 5, and RNA selectively precipitated with ethanol (5). RNA pellets were dissolved, heated, and aliquots spotted onto nitrocellulose, as described (5). Prehybridization, hybridization, and washes were as described above.

**Results**

**Type II Collagen mRNA during Chondrogenesis**

In our initial studies we examined changes in the cytoplasmic levels of type II collagen mRNA during the course of the chondrogenic differentiation of the distal subridge mesenchymal cells of stage 25 wing buds in micromass culture. Stage 25 subridge mesoderm consists of a relatively homogeneous population of undifferentiated chondrogenic progenitor cells, which uniformly progress through the phases of chondrogenesis in micromass culture and form a virtually uniform sheet of cartilage with little, if any, non-chondrogenic tissue detectable (10). In such cultures, widespread prechondrogenic aggregations of cells are detected by the end of the first day of culture, after which there is a progressive and uniform accumulation of Alcian blue-positive cartilage matrix by the cells (10).

At 3 h after the initiation of culture, which is well before any morphological indications of differentiation by the mesenchymal cells, low but clearly detectable levels of RNA sequences that hybridize to the cloned type II collagen cDNA probes are present in the cytoplasm of the cells (Fig. 1). By 20 h, which corresponds to the onset of overt chondrogenesis as characterized by the formation of extensive prechondrogenic aggregations of cells throughout the culture, there is a greater than seven-fold increase in the level of hybridizable type II collagen mRNA sequences in the cytoplasm above that detectable at 3 h. During the subsequent 3 d of culture, there is a continuous and progressive increase in the accumulation of cytoplasmic type II collagen mRNA which parallels the progressive accumulation of cartilage matrix by the cells. By the fourth day of culture there has been about a 100-fold increase in the amount of cytoplasmic type II collagen mRNA per cell over that which was detectable at 3 h. A virtually identical pattern of accumulation of cytoplasmic type II collagen mRNA is observed using either type II collagen cDNA plasmid pCAR2 or pCAR1. No hybridization to the cloned rat growth hormone cDNA plasmid, GH1-pBR322 (13), is detectable at any time during the culture period, indicating that none of the hybridization detectable with the type II collagen cDNA probes is due to the nonspecific binding of plasmid DNA sequences.

The presence of low but clearly detectable levels of RNA sequences complementary to cloned type II collagen cDNA probes at 3 h after the initiation of culture suggested that the type II collagen gene is being expressed at low levels before the onset of overt differentiation. We have obtained further evidence for this by demonstrating that hybridizable type II collagen RNA sequences are present in the cytoplasm of the prechondrogenic mesenchymal cells in the distal subridge region of the stage 25 wind bud (Fig. 2). It should be emphasized that the distal subridge region at stage 25 consists of a homogeneously appearing population of cytologically unspecialized mesenchymal cells.
enanchymal cells (19, 30). The cells synthesize high levels of hyaluronate (20) and type I collagen, but do not synthesize detectable amounts of type II collagen (28, 29). The amount of cytoplasmic type II collagen mRNA in these prechondrogenic mesenchymal cells is 0.5–1.5% of the amount we detect in the cytoplasm of the chondrocytes of 7-d limb cartilage or 12-d sternal cartilage.

In an attempt to determine how early in limb development the type II collagen gene is expressed, we looked for the presence of hybridizable type II collagen RNA in cytoplasmic extracts prepared from the mesenchymal cells comprising the wing buds of stage 18/19 embryos, which is shortly after the initial formation of the limb bud. As shown in Fig. 2, RNA sequences complementary to the type II collagen cDNA probes are clearly detectable in the cytoplasm of prechondrogenic limb mesenchymal cells even at this early stage of development. In fact, the amount of hybridizable type II collagen mRNA per cell is consistently somewhat greater (1.5–2 times) in stage 18/19 mesenchymal cells than in stage 25 subridge mesenchymal cells (Table I). In contrast, there is virtually no detectable hybridization of the type II cDNA probes to cytoplasmic extracts of a clonal cell line of rat pituitary GH3 cells (Fig. 2), which served as a negative control.

To confirm the results obtained using cytoplasmic dot hybridization, we purified RNA from stage 18/19 limb mesenchymal cells, stage 25 subridge mesenchymal cells, and GH3 cells using the guanidine–HCl extraction/ethanol precipitation procedure of Cheley and Anderson (5), and analyzed the RNA by dot-blot hybridization. Type II collagen mRNA sequences are clearly detectable in the RNA isolated from the prechondrogenic limb mesenchymal cells at either stage, whereas no hybridization is detectable to RNA extracted from GH3 cells (Fig. 2). The quantitative results we obtained using cytoplasmic dot hybridization or dot-blot hybridization of purified RNA are quite comparable (Table I).

Since prechondrogenic limb mesenchymal cells synthesize type I collagen and the sequence homology between types I and II collagens is quite high, it was crucial to eliminate the
possibility that the relatively low hybridization signal detected with type II collagen cDNA in these cells might be due to cross-hybridization of the type II cDNA probe to type I collagen mRNA sequences. In this regard, it should be emphasized that our hybridizations and washes were performed under very stringent conditions, i.e. hybridizations being done in 50% formamide at 55°C, and multiple washes being done in 0.1X SSC at 55°C. These conditions are, in general, as stringent as those that several investigators have shown prevent cross-hybridization between several type II collagen cDNAs and type I collagen mRNAs, or between type I collagen cDNAs and type II collagen mRNAs (8, 9, 26, 37). To directly test the possibility of cross-hybridization, aliquots of cytoplasmic extracts of stage 18/19 and stage 25 subridge mesenchymal cells and aliquots of purified calvaria RNA, which contains large quantities of both α1(I) and α2(I) mRNAs, were hybridized with the type II collagen cDNA probe. In parallel experiments, equivalent aliquots of the cytoplasmic extracts and calvaria RNA were hybridized with either α1(I) or α2(I) cDNA probes. As shown in Fig. 3, under conditions in which type II cDNA hybridizes to cytoplasmic RNA sequences in limb mesenchymal cells, there is no detectable hybridization of the type II probe to an amount of calvaria RNA which contains 4-5 times more hybridizable α1(I) and α2(I) mRNA sequences than do cytoplasmic extracts of limb mesenchymal cells. This demonstrates that the hybridization of the type II cDNA probe to sequences in prechondrogenic mesenchymal cells is not due to cross-hybridization of the probe to α1(I) or α2(I) mRNA sequences, or to other RNA species present in total calvaria RNA.

**Type I Collagen mRNA during Chondrogenesis**

Changes in the cytoplasmic levels of mRNAs for the α1(I) and α2(I) chains of type I collagen during the chondrogenic differentiation of stage 25 subridge mesenchymal cells in micromass culture are summarized in Table II. There is not a progressive decline in the accumulation of type I collagen mRNAs during the course of chondrogenesis as we had anticipated. Rather, the relatively high levels of cytoplasmic type I collagen mRNAs present during the early culture period persist, and, in fact, there is an increase in the level of type I mRNAs during the course of in vitro cartilage differentiation (Table II). This was unexpected, since at the end of the culture period few, if any, nonchondrogenic cells are detectable, and little type I collagen production would be expected to be occurring. It is conceivable, however, that some type I collagen-producing fibrogenic cells differentiated in the cultures even though such cells are not readily detectable morphologically.

We, therefore, examined the levels of type I collagen mRNAs in the purified chondrocytes comprising 7-d limb cartilage and 12-d sternal cartilage, since these cartilages do not synthesize immunologically or biochemically detectable amounts of type I collagen (36). As shown in Fig. 4 and Table III, substantial levels of α1(I) and α2(I) mRNA sequences are present in the cytoplasm of the differentiated chondrocytes of both limb and sternal cartilage. The levels of α1(I) and α2(I) mRNAs per cell in limb and sternal cartilage are comparable to the levels found in prechondrogenic limb mesenchymal cells which do synthesize type I collagen (Table III). By comparing the relative hybridization of the α1(I) cDNA probe and the α2(I) cDNA probe to equal amounts of purified calvaria RNA and to equal cytoplasmic aliquots of limb and

Table II. Cytoplasmic Type I Collagen mRNA Accumulation during the Course of the Chondrogenic Differentiation of Subridge Limb Mesenchymal Cells in Micromass Culture

<table>
<thead>
<tr>
<th>Hours in culture</th>
<th>α2(I) (mRNA/μg DNA)</th>
<th>α1(I) (mRNA/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>745 582</td>
<td>290</td>
</tr>
<tr>
<td>20</td>
<td>349 324</td>
<td>200</td>
</tr>
<tr>
<td>48</td>
<td>417 742</td>
<td>525</td>
</tr>
<tr>
<td>72</td>
<td>783 613</td>
<td>578</td>
</tr>
<tr>
<td>96</td>
<td>1,000 1,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>

The levels of mRNA were quantified by scanning densitometry. In each experiment, the amount of mRNA/μg DNA at each timepoint is expressed as an amount relative to that at 96 h of culture, which was arbitrarily set at 1,000. Note that the normalized α2(I) and α1(I) mRNA levels cannot be directly compared.

Figure 3. (A) Autoradiographs comparing hybridization of type II collagen cDNA (α1[II]) and α2(I) cDNA to cytoplasmic aliquots of stage 18/19 mesenchymal cells (18) (3.4 × 10⁵ cell equivalents), stage 25 subridge mesenchymal cells (25) (6.8 × 10⁵ cell equivalents), and to 0.8 μg (CV-1) and 0.4 μg (CV-2) of calvaria RNA. (B) Hybridization of type II collagen cDNA (α1[II]) and α1(I) cDNA to cytoplasmic aliquots of stage 18/19 (18) (3.2 × 10⁵ cell equivalents) and stage 25 subridge (25) (6.4 × 10⁵ cell equivalents) mesenchymal cells and to 0.4 μg (CV-1) and 0.1 μg (CV-2) of calvaria RNA.
Table III. The Amount of Type I Collagen mRNAs in the Cytoplasm of Differentiated Limb and Sternal Chondrocytes Compared to Prechondrogenic Mesenchymal Cells

<table>
<thead>
<tr>
<th>mRNA/μg DNA</th>
<th>mRNA</th>
<th>Limb mesenchyme</th>
<th>7-d limb cartilage</th>
<th>12-d sternal cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2 (I)</td>
<td>10</td>
<td>12.9</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>α1 (I)</td>
<td>10</td>
<td>14.3</td>
<td>10.4</td>
<td></td>
</tr>
</tbody>
</table>

The prechondrogenic mesenchymal cells are those comprising the distal subridge region of stage 25 wing buds. The levels of mRNA were determined by scanning densitometry. The amounts of α2 (I) or α1 (I) mRNA/μg DNA in the chondrocytes is expressed as an amount relative to that in the mesenchymal cells, which was arbitrarily set to 10. Note that the normalized α2 (I) and α1 (I) mRNA levels cannot be directly compared.

The prechondrogenic mesenchymal cells in the cytoplasm of the chondrocytes can be estimated, since it is known that the α1(I)/α2(I) mRNA ratio in calvaria is 2:1 (39). The cytoplasmatic α1(I)/α2(I) mRNA ratio in limb and sternal cartilage is about 0.4 and 0.75, respectively. Thus in differentiated chondrocytes which do not synthesize type I collagen, there is a large excess of cytoplasmic α2(I) mRNAs relative to α1(I) mRNAs, which contrasts to the α1(I)/α2(I) mRNA ratio of 2:1 in calvaria. Interestingly, the cytoplasmic α1(I)/α2(I) mRNA ratio is also not 2:1 in prechondrogenic limb mesenchymal cells. In stage 18/19 limb mesenchymal cells the α1(I)/α2(I) mRNA ratio is ~1:1; and in stage 25 subridge mesenchymal cells the ratio is 1:3.

Discussion

Our studies on the accumulation of cytoplasmatic type II collagen mRNA during the progression of limb cartilage differentiation in vivo and in vitro have revealed several interesting aspects of the regulation of collagen gene activity during the process. First of all, a striking increase in the amount of cytoplasmatic type II collagen mRNA is detectable coincident with the crucial condensation or aggregation phase of chondrogenesis in vitro, in which prechondrogenic mesenchymal cells become closely juxtaposed to one another before depositing a cartilage matrix. This temporal correlation suggests that this change in type II collagen gene activity occurs as a consequence of regulatory events taking place during the condensation process. Among those interrelated events that have previously been shown to be involved in triggering chondrogenesis during condensation are cell–cell and/or cell–matrix interactions; cytoskeletal-linked change in cell shape; and prostaglandin–mediated elevations in cellular cAMP levels (see the introduction).

After the crucial condensation process, there is a continuous and progressive increase in the accumulation of cytoplasmatic type II collagen mRNA which parallels the progressive accumulation of cartilage matrix by the cells. This observation is in agreement with the recent study of Kravis and Upholt (22) on type II collagen mRNA levels in whole limb buds and in high density cultures prepared from whole limb buds. These results indicate that the expression of the type II collagen gene during limb cartilage differentiation is regulated primarily by mechanisms controlling the rate of accumulation of type II collagen mRNA. It is quite likely that greatly accelerated transcription of the type II collagen gene is largely responsible for this increase in mRNA accumulation, although accelerated processing of nuclear transcripts and/or an increase in cytoplasmic mRNA stability might also conceivably be involved.

It is important to emphasize, however, that the onset of overt cartilage differentiation apparently does not involve the initiation or activation of the transcription of the type II collagen gene. We unexpectedly found low, but clearly detectable levels of type II collagen mRNA in the cytoplasm of the prechondrogenic mesenchymal cells comprising stage 18/19 wing buds and the distal subridge region of stage 25 wing buds. Low levels of type II mRNA have also been detected in stage 20 limb buds (22). Thus the type II collagen gene is being expressed at low levels in limb mesenchymal cells at the earliest stages of limb development, well before the onset of overt cartilage differentiation and the accumulation of detectable amounts of type II collagen. It is possible that type II collagen mRNAs are being translated in prechondrogenic mesenchymal cells, but that the amount of type II collagen that accumulates is below the level of detection of the immunological and biochemical procedures that have been used to study its production. In this regard, at least a portion of the type II collagen mRNA in stage 20 limb buds is associated with polysomes (22). Alternately, it is possible that posttranscriptional regulatory controls may limit the translation of type II collagen mRNA before the overt differentiation of the cells. In any event, the low level expression of the type II collagen gene by chondrogenic progenitor cells before the onset of overt differentiation may represent a molecular manifestation of the state of determination of the cells. It is conceivable that the presence of cytoplasmatic type II collagen mRNA might be useful as a molecular marker to elucidate when and where during embryogenesis determination of a specific limb cell type occurs. The precocious expression of muscle-specific actin genes has served as a molecular marker for detecting the onset of muscle cell determination in the amphibian embryo (27).

Our results indicate that type I collagen gene expression during chondrogenesis is regulated, at least in part, at the translational level. Substantial amounts of mRNAs for the α1(I) and α2(I) chains of type I collagen are present in the cytoplasm of the well-differentiated chondrocytes comprising 7-d limb cartilage and 12-d sternal cartilage, which do not synthesize detectable amounts of type I collagen. The levels of cytoplasmatic type I collagen mRNAs in cell in these cartilage tissues is comparable to the levels found in prechondrogenic mesenchymal cells. Although it is difficult to unequivocally eliminate the possibility, it is highly unlikely that a small number of potentially contaminating perichondrial cells could account for the substantial levels of type I mRNAs in our cartilage preparations. Evidence for the translational regulation of type I collagen gene expression in chondrocytes has also recently been provided by other investigators. Type I collagen mRNAs have been detected in limb and sternal cartilage (22); in the cytoplasm of cultured sternal chondrocytes (31); and in cultured vertebral chondrocytes, which also do not synthesize type I collagen (1, 9).

We have also found that in differentiated chondrocytes, which are not producing type I collagen, there is an excess of α2(I) mRNAs relative to α1(I) mRNAs in cytoplasm. This contrasts to the α1(I)/α2(I) mRNA ratio of 2:1 in embryonic chick calvaria that are synthesizing large quantities of type I.
collagen (39). Our finding is consistent with the recent observation of Saxe et al. (31) that cultured sternal chondrocytes contain substantial quantities of a2(II) mRNAs in their cytoplasm, but only small quantities of a1(II) mRNAs. Interestingly, our results indicate that the cytoplasmic a1(II)/a2(II) mRNA ratio is also not 2:1 in prechondrogenic mesenchymal cells, the a1(II)/a2(II) ratio being ~1:1 in stage 18/19 limb mesenchymal cells and ~1:3 in stage 25 subcortical mesenchymal cells. It will be important to determine if the decrease in the a1(II)/a2(II) mRNA ratio in stage 25 subcortical cells compared to stage 18/19 limb mesenchymal cells reflects differences in the rate of type I collagen production in these progenitor cells.

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