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Collagen gene expression during chondrogenesis from chick periosteum-derived cells

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Chick periosteum-derived cells, which do not enter the chondrogenic cell lineage during normal bone development and growth, exhibit chondrogenic potential in high cell density culture conditions. In such cultures, collagen gene expression was temporally analyzed at the mRNA level by a reverse transcription PCR (RT-PCR) procedure, which showed that α1(II) and α1(IX) collagen mRNAs are coordinately increased, coincident with the onset of overt chondrogenesis, and subsequently decreased as chondrocytes exhibited hypertrophic characteristics. α1(X) collagen mRNA was detected well before the onset of chondrogenesis and markedly increased along with the hypertrophic change. For α2(I) collagen, both the bone/tendon form and the cartilage form of mRNA were detected throughout the culture period. This culture system provides an experimental vehicle capable of investigating the molecular events involved in the full range of chondrogenic differentiation starting from uncommitted periosteum-derived mesenchymal stem cells.

Periosteum-derived cell; Collagen; Gene expression; Cartilage differentiation; Chondrocyte

1. INTRODUCTION

The periosteum, the outer layer of bone, plays a crucial role in the radial growth of long bone; mesenchymal stem cells [1] in the periosteum differentiate directly to osteoblasts and never enter a chondrogenic lineage in normal long bone development and growth. In fracture healing, however, periosteum-derived mesenchymal cells differentiate into chondrocytes with subsequent endochondral bone formation [2]. Thus, cells in the periosteum appear to possess the potential to differentiate into not only osteoblasts but also chondrocytes under certain conditions [3]. In this regard, we have previously reported that chick periosteum-derived mesenchymal cells, when plated at a high cell density in culture, exhibit chondro-osteogenic phenotypes in vitro [4]. Many cell culture systems which induce chondrogenesis in vitro have been reported [5–7]; however, these are cultures of cells which are already committed to differentiate into chondrocytes. Our culture system is of interest because chondrogenesis occurs from undifferentiated cells which are not committed to chondrocytes in the normal course of bone physiology or development. To investigate the differentiation of chondrocytes and the formation of hypertrophic cartilage from uncommitted periosteum-derived cells at the molecular level, we have used this periosteum-derived mesenchymal cell culture system. In this report, the temporal changes are presented for type I, II, IX and X collagen mRNAs during an early phase of chondrogenesis from chick periosteum-derived cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Periosteal cells were prepared as described elsewhere [4]. Briefly, tibial periostea of 1-week-old White Leghorn chicks were digested with collagenase–trypsin and the liberated cells were sparsely plated at a density of 1.0×10^6 cells per 100 mm plastic tissue culture dish in BGJb medium (Gibco Lab.) supplemented with 10% fetal calf serum (FCS) (Gibco Lab.). After 10–12 days of primary culture, when these cells reached confluence, they were replated at a density of 1.0×10^5 cells per 50 μl micromass (high cell density culture [8]). The day of introduction into the high density culture was designated as day 0. BGJb medium supplemented with 10% FCS was changed every other day; starting on day 1, freshly prepared ascorbic acid (sodium ascorbate, Sigma Chemical Co.) was added daily at a final concentration of 50 μg/ml. For light microscopic observation, cell cultures were peeled off the dishes and processed for paraffin sections perpendicular to the culture dishes. These sections were stained with Toluidine blue (0.1%, pH 7.0); some sections were treated with von Kossa stain followed by counterstaining with Toluidine blue.

2.2. *Cytoplasmic RNA extraction and reverse transcription-PCR (RT-PCR) analysis*

Cytoplasmic RNA was extracted from the cultured cells on day 5 of the primary culture and day 2, 4, 6, 9, 12 and 15 of the secondary high density culture with a Cytoplasmic RNA Extraction Kit (5 Prime-3 Prime Inc., Pennsylvania). As a control, cytoplasmic RNA was also extracted from chick muscle fibroblasts, skin fibroblasts and sternal chondrocytes of 15-day embryos, and liver.

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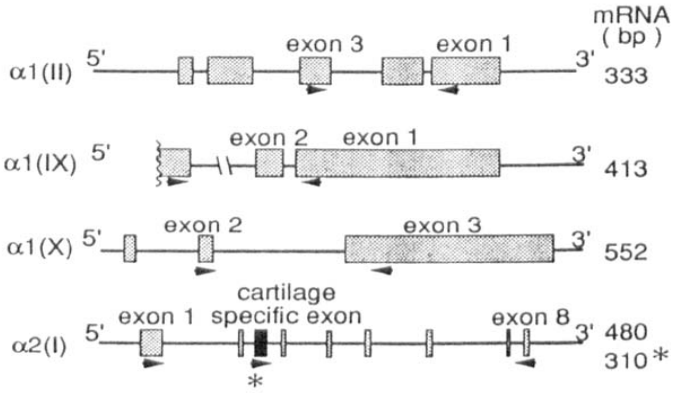
500 ng of cytoplasmic RNA was reverse transcribed to make cDNA with random hexamer as a primer following a previously described method [9]. PCR was performed with 3 μl out of 80 μl of the cDNA aliquot as a template with *Thermus thermophilus* (Tth) DNA polymerase (Toyobo Co., Osaka). A combination of 5′ and 3′ primers derived from separate exons was used to detect specific collagen mRNAs (Fig. 1). For α2(I) procollagen, two different 5′ primers were prepared to detect both the bone/tendon form of mRNA and the cartilage form of mRNA [10]. In preliminary experiments, various PCR conditions were tested with different amounts of cytoplasmic RNA from sternal chondrocytes to find the conditions wherein different amounts of collagen mRNA proportionally affect the yield of PCR products. The following conditions were adopted in the present study: 42 cycles performed with Zymoreactor (Atto Co., Tokyo) at 94°C for 1 min, 61°C for 2 min, 72°C for 3 min and 72°C for 6 min at the end of the cycles with 5 pmol of each pair of primers and 1.5 mM $MgCl_2$. Equal aliquots of PCR products were then separated on an agarose gel. Southern blot analysis of the PCR products were performed as described previously [11]. The cDNA clones, pYN2142(α1(II)) [12], pYN1738(α1(IX)) [13] and pYN92E(α1(X)) [14] were used for hybridization.

3. RESULTS

3.1. *Transition of cell culture*

The cellular and morphological transitions of high cell density cultures have been described in detail elsewhere [4]. In brief, periosteum-derived mesenchymal cells in primary culture appeared undifferentiated and homogeneous, a population of fibroblast-like cells (Fig. 2A). These cells did not express chondrogenic or osteogenic phenotypes as judged by their morphology and



B

α1(II)	5' sense	5'-GCAGAGACCATCAACGGCGGT-3'
	3' antisense	5'-CAGGCGCGAGGTCTTCTGCGA-3'
α1(IX)	5' sense	5'-CAGCTGGCAGCCAGTCTTAGG-3'
	3' antisense	5'-CTCTGTCCAGCCTGCATTCGG-3'
α1(X)	5' sense	5'-AAGGGGCCACCACACTTTCTA-3'
	3' antisense	5'-TTCTCCAGGCTTCCCTATCCC-3'
α2(I)	5' sense	5'-TTACTCCTCGCGACTGTATGC-3'
	*5' sense	5'-TGCACGGAAGGATTATGAGAG-3'
	3' antisense	5'-GCTCACCAGGAACACCTTGAA-3'

Fig. 1. Primers used for RT-PCR analysis of collagen mRNAs. (A) The shaded boxes indicate exons and lines indicate introns of each collagen gene. The exons are numbered from the 3′ end in α1(II) and α1(IX) collagen genes and from the 5′ end in α1(X) and α2(I) collagen genes. The arrowheads indicate approximate locations of synthetic oligonucleotide primers used in this study. The numbers on the right show the predicted lengths of RT-PCR products from each collagen mRNA. The primer with the asterisk was prepared within the cartilage-specific exon and the number with the asterisk is the predicted length of the RT-PCR product from the cartilage form of α2(I) mRNA. (B) The sequences of the primers were selected from published data [12,13,21–23].

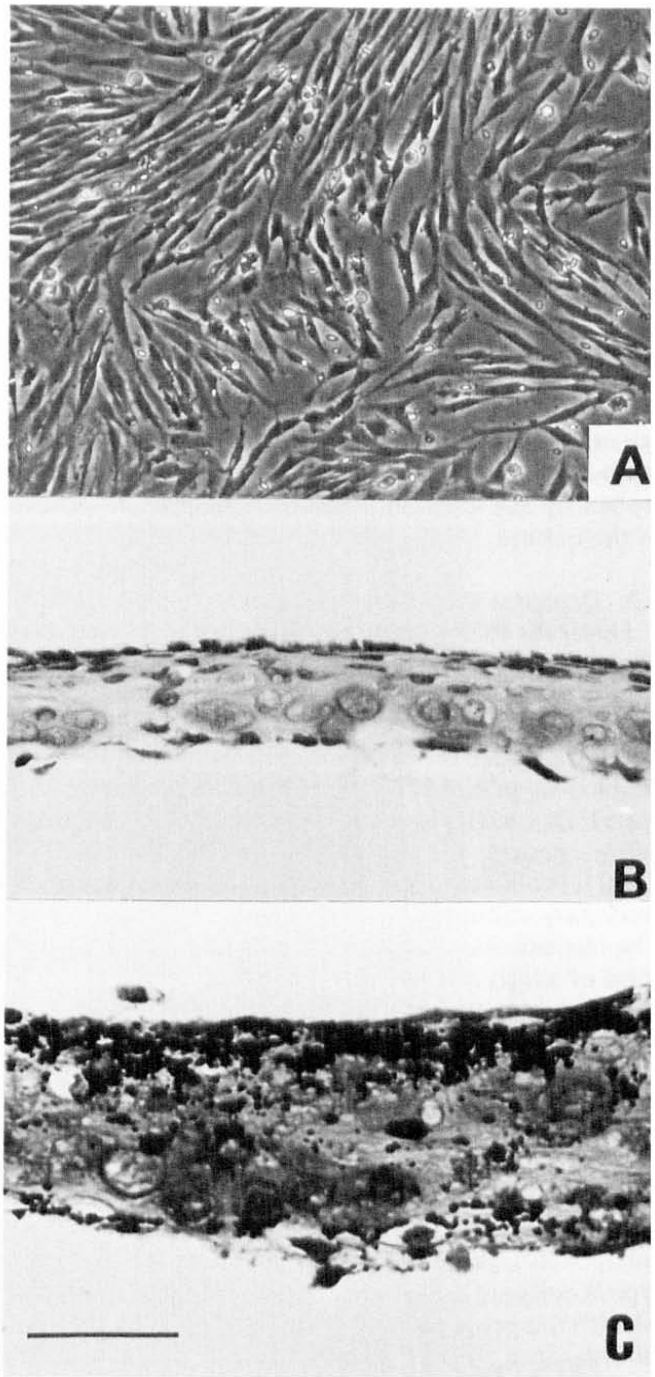


Fig. 2. Morphologic appearance of the culture. (A) Phase photomicrograph of periosteum-derived mesenchymal cells in the primary culture. (B) Perpendicular section of day 9 of the secondary high density culture (Toluidine blue staining). (C) Perpendicular section of day 15 culture (von Kossa stain counterstained with Toluidine blue). Bar in C = 50 μm.

immunohistochemical study of antigens specific to chondrocytes or osteoblasts [4]. When introduced into high cell density culture, these cells first formed a multilayer of fibroblast-like cells, the majority of which differentiated into chondrocytes (Fig. 2B) and further developed into hypertrophic chondrocytes (Fig. 2C). Appearance of type II and type X collagen in the transition of this culture has been immunohistochemically detected as reported previously [4]. The top layer of the culture differentiated into bone tissue, as evidenced by the mineralization (Fig. 2C) and immunohistochemical reactivity to bone Gla protein and osteocyte-specific probes (see ref. [4]). These findings indicate that undifferentiated mesenchymal cells from periosteum have the potential to terminally differentiate into either hypertrophic chondrocytes or osteoblasts/osteocytes as controlled by the local cues due to their relative positions in the culture.

3.2. *Detection of collagen mRNAs*

Using the PCR conditions described in Materials and Methods, titered α1(IX) collagen mRNA from sternal chondrocytes was proportionally amplified (Fig. 3). α1(II) and α1(X) collagen mRNA were also linearly amplified under the same conditions (not shown). This enabled us to compare the relative level of mRNA for the α1(IX), α1(II) and α1(X) chain at each phase of the culture period.

α1(II) collagen mRNA was first observed on day 9 of secondary culture as a band of predicted size (Fig. 4A). The identification of the PCR product as the amplified form of α1(II) mRNA was confirmed by the restriction mapping (not shown) and Southern Blot analysis (Fig. 5A). By the Southern blot analysis, small amounts of α1(II) mRNA were detectable starting on day 4. Compared to the level of β-actin mRNA, which is almost constant among the different time points (Fig. 4F), type II collagen mRNA increased markedly until day 9, coincident with the onset of overt chondrogenesis and the appearance of positive immunostaining for type II collagen as reported in a previous study [4]. Thereafter, type II collagen mRNA decreased until day 15, in parallel with the progress of chondrocyte hypertrophy in the culture. In a similar fashion, α1(IX) collagen mRNA was first detected on day 4 by Southern blot analysis (Fig. 5B), reached a peak on day 9, and then decreased. Although the α1(II) and α1(IX) collagen genes were coordinately expressed during the chondrocyte differentiation in this culture system, no α1(IX) mRNA was detected in the control muscle and skin fibroblasts, whereas a small amount of α1(II) mRNA was detectable in these cells (Fig. 5A,B).

Type X collagen gene expression was also analyzed by RT-PCR and Southern blot. Unexpectedly, type X collagen mRNA was detectable throughout the 15-day secondary high density culture period (Figs. 4C and 5C). Although type X collagen mRNA was absent in the primary culture, it was detected as early as day 2 of the high density secondary culture, which is well before the onset of chondrogenesis. Type X collagen mRNA increased along with the chondrocyte hypertrophy. Type X collagen mRNA was not detectable in muscle fibroblasts, skin fibroblasts or liver (Figs. 4C and 5C).

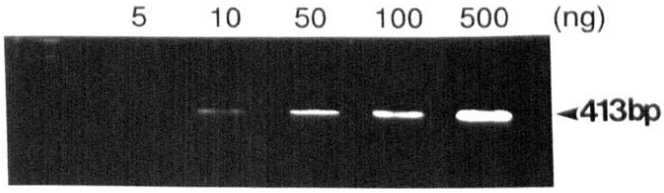


Fig. 3. Agarose gel electrophoresis of RT-PCR products stained with ethidium bromide. Different amounts of control sternal chondrocyte RNA were reverse transcribed and amplified by PCR with the α1(IX) collagen-specific primer. The number above each lane indicates the amount of sternal chondrocyte RNA used. Note that different amounts of RT-PCR products were obtained and these amounts were proportional to the imput amount of sternal chondrocyte RNA.

For α2(I) collagen, the expression of the two forms of mRNA, namely the bone/tendon form and the cartilage form, was analyzed. The bone/tendon form of α2(I) collagen mRNA was detected throughout the whole culture period (Fig. 4D); this form of mRNA was present in control muscle fibroblasts, skin fibroblasts, and liver, but not in sternal chondrocytes. The cartilage form of α2(I) mRNA, which is a major α2(I) transcript in cartilaginous tissue [10], was also detectable throughout the culture period (Fig. 4E). The presence of the cartilage form of α2(I) mRNA was not restricted to cartilage, but was widely distributed among non-cartilaginous tissues (Fig. 4E).

4. DISCUSSION

The present study confirms that α1(II) and α1(IX) collagen gene expression is associated with differentiation of chondrocytes. As speculated previously [15], this co-expression of types II and IX collagen mRNAs may be required for the appropriate assembly of thin fibrils in the cartilaginous extracellular matrix. The initiation of production of these mRNA species on day 4 of the secondary culture, which is about 4 days before the onset of overt chondrogenesis, may indicate the time around which uncommitted bipotential progenitor cells in the periosteum commit to a chondrogenic lineage. It should be noted, however, that the presence of a small amount of α1(II) mRNA is non-chondrogenic cells may indicate the presence of alternatively spliced mRNA which exists in pre-chondrogenic cells [16].

To our surprise, type X collagen mRNA was detectable throughout the secondary high density culture period, while, in our previous study, immunoreactive type X collagen was detectable only after day 12. These results suggest that in the present culture system the periosteum-derived cells may express type X collagen

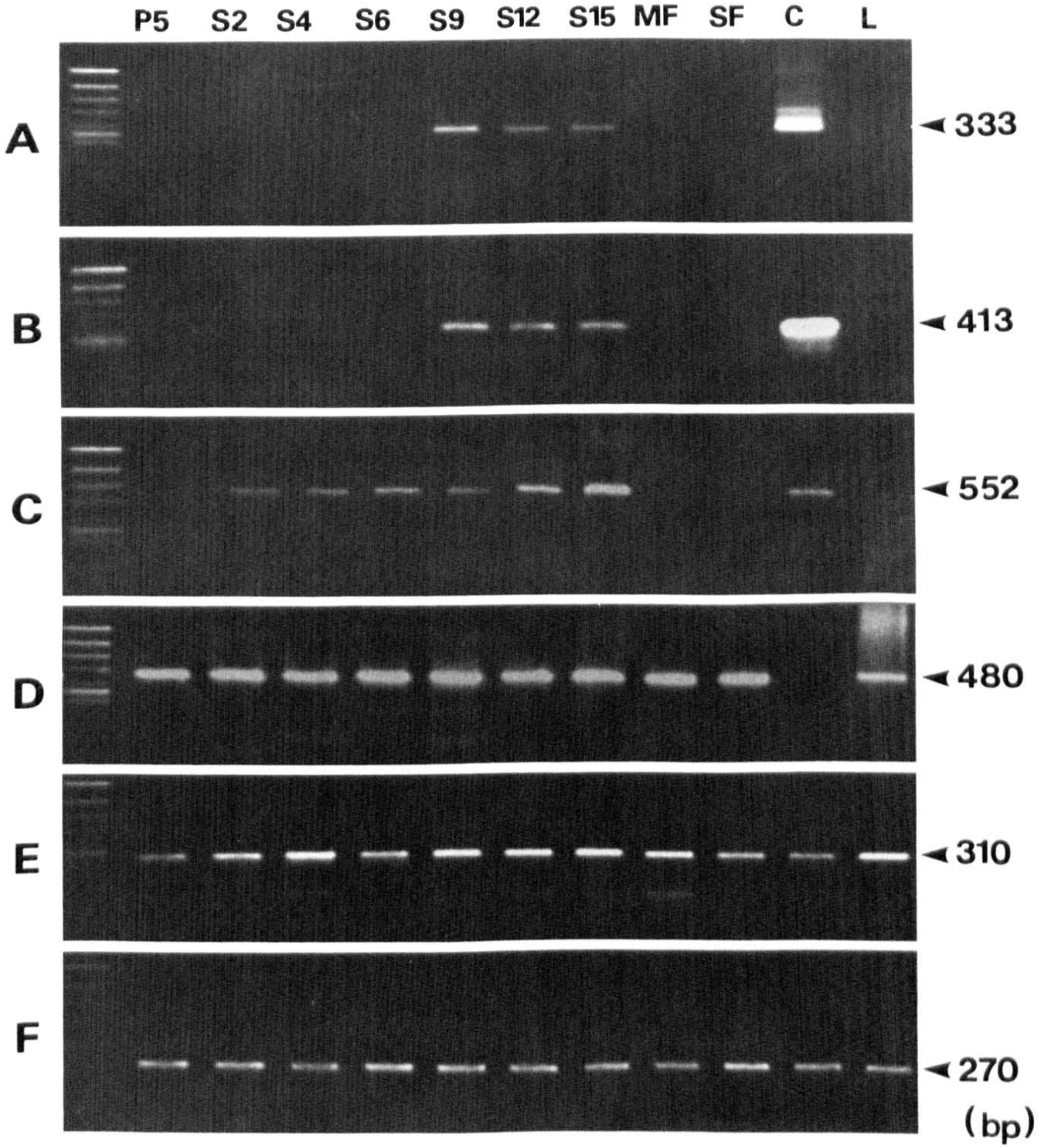


Fig. 4. Agarose gel electrophoresis of RT-PCR products stained with ethidium bromide. Total RNA was isolated from day 5 primary cultures (P5), and from day 2 (S2), day 4 (S4), day 6 (S6), day 9 (S9), day 12 (S12) and day 15 (S15) secondary cultures, muscle fibroblasts (MF), skin fibroblasts (SF), sternal chondrocytes of 15-day embryos (C), and liver (L). RNA samples were amplified with primers specific for (A) α1(II) collagen, (B) α1(IX) collagen, (C) α1(X) collagen, (D) the bone/tendon form of α2(I) collagen, (E) the cartilage form of α2(I) collagen and (F) β actin.

gene before the apparent onset of chondrocyte hypertrophy. During chondrocyte hypertrophy up to day 15 of culture, type X collagen mRNA increased. On the other hand, the mRNA for α1(II) and α1(IX) collagen diminished. Similar results have been reported in the hypertrophic cartilage of chick tibiotarsus and for chondrocytes in suspension culture [15,17,18]. These observations together suggest that a shift occurs from

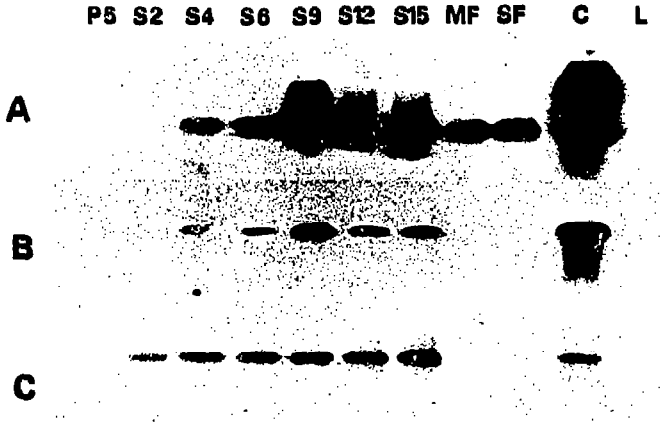


Fig. 5. Southern blot analysis of RT-PCR products. Gels in Fig. 4A, 4B and 4C were blotted onto nylon membranes and hybridized with cDNA probes for (A) α1(II) collagen, (B) α1(IX) collagen and (C) α1(X) collagen.

type II and type IX collagen to type X collagen when chondrocytes become hypertrophic. Although the type X collagen mRNA was detected before the appearance of type II collagen mRNA, the earlier appearance of type X relative to type II collagen gene expression must await further analysis, because the efficiency of amplification in RT-PCR with different primers is not equivalent.

In this study with RT-PCR, both the bone/tendon form and the cartilage form of α2(I) collagen mRNA were detectable throughout the culture period. The bone/tendon form was absent only in sternal chondrocytes. These results suggest that the 'cartilage-specific' promoter is widely utilized in various tissues other than cartilage. In addition, the inability of sternal chondrocytes to synthesize the α2 subunit of type I collagen [19,20] might result from complete repression of the bone/tendon form of α2(I) mRNA.

It is important to point out that our culture system might reflect the in vivo situation for fracture healing. In fracture healing, the cells (possibly a population of multipotential mesenchymal stem cells) in the periosteum are believed to proliferate, migrate into the fracture site, and differentiate into chondrocytes which are subsequently replaced by bone. This culture system follows most of this sequence in vitro. The intrinsic and extrinsic factors which govern the chondrogenic or osteogenic developmental potential of periosteum-derived cells are the subject of future experimentation.

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