

Changes in the Expression of Collagen Genes Show Two Stages in Chondrocyte Differentiation In Vitro

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Abstract. This report deals with the quantitation of both mRNA and transcription activity of type I collagen gene and of three cartilage-specific collagens (types II, IX, and X) during in vitro differentiation of chick chondrocytes. Differentiation was obtained by transferal to suspension culture of dedifferentiated cells passaged for 3 wk as adherent cells. The type I collagen mRNA, highly represented in the dedifferentiated cells, rapidly decreased during chondrocyte differentiation. On the contrary, types II and IX collagen mRNAs sharply increased within the first week of suspension culture, peaked in the second week, and thereafter began to decrease. This decrease was particularly significant for type IX collagen mRNA. The level of type X collagen mRNA progressively increased during the

course of the culture, reached its maximal value after 3–4 wk, and decreased only at a later stage of cell differentiation.

As determined by in vitro run-off transcription assays, all these changes in collagen mRNA levels could be attributed to parallel modifications in the relative rate of transcription of the corresponding collagen genes.

We suggest that chicken chondrocyte differentiation proceeds through at least two different steps: (a) first, transition from a stage characterized by a high level of type I collagen mRNA to a stage characterized by predominance of types II and IX collagen mRNAs; (b) later, transition to a stage characterized by the highest level of type X collagen mRNA.

ORGANOGENESIS of long bones involves cellular differentiation and continuous synthesis and remodeling of the extracellular matrix. During the first stages of chick development, mesenchymal cells in the limb buds differentiate, possibly through distinct regulatory steps (22), and condense to form a blastema from which cartilaginous bones develop. This process is characterized by profound changes in the cell morphology accompanied by the synthesis of novel extracellular macromolecules, among which are cartilage-specific proteoglycans and collagens (7). Changes in the levels of types I and II collagens and of their mRNAs during chondrogenesis in vivo and in vitro were detected with the use of specific antibodies and cDNA probes (27, 10, 11). Cartilaginous bones grow because of cell proliferation and deposition of new extracellular matrix. Proliferating chondrocytes progressively mature into hypertrophic non-dividing cells. Hypertrophy of chondrocytes, first detectable in the diaphysis and after in the epiphyseal regions, is accompanied by the onset of type X collagen synthesis (2, 20). Hypertrophic cartilage undergoes calcification, is invaded by blood vessels and osteogenic cells, and is replaced by bone tissue (18). To investigate at the molecular level the differentiation of hypertrophic cartilage chondrocytes we have used an in vitro system starting with tibial chondrocytes of early stage chick embryos (3). In this system, chondrocytes obtained by enzymatic dissociation of stage 28–30 (9) embryo tibiae, passaged on tissue culture dishes, assume a fibroblast-like

morphology and switch from type II to type I collagen synthesis. When these adherent cells are transferred to agarose-coated dishes (suspension culture) they revert to the chondrocytic phenotype, and, after a transient stage in which cells form aggregates, they mature to single hypertrophic chondrocytes that produce collagen type X (3).

We have also shown that the addition of ascorbic acid to the suspension culture results in the organization of the extracellular matrix and in the maturation of the cell aggregates into hypertrophic cartilage (23). In this paper we report the variations in the steady-state levels of types I, II, IX, and X collagen mRNAs and the transcriptional activity of the corresponding genes during the in vitro differentiation of chick embryo tibial chondrocytes starting from adherent cells that produce type I collagen. As in vivo studies on mRNA levels during limb bud development (10, 11), we have found a rapid decrease of the type I collagen mRNA and a concomitant and rapid increase of the type II collagen mRNA in the first 2 wk of culture. At the same time the mRNA for the type IX collagen increased. The increase of the type X collagen mRNA was more progressive and continuous, and at later times of culture was simultaneous to the decrease of types II and IX collagen mRNAs. All these changes in collagen mRNA levels could be correlated to parallel changes in the transcription rate of the specific genes. These findings lead us to suggest that, during the process of hypertrophic chondrocyte differentiation, a shift in production from type I to

types II and IX collagens occurs when chondrogenesis begins, and a shift from types II and IX to type X collagens occurs when chondrocytes become hypertrophic.

Materials and Methods

Cell Culture

Adherent dedifferentiated and differentiating chondrocytes were obtained as described by Castagnola et al. (3). Briefly, dedifferentiated chondrocytes were obtained by plating freshly dissociated chondrocytes from tibiae of stage 28–30 chick embryos (9) in plastic tissue culture dishes.

Differentiating chondrocytes were obtained by transferring the dedifferentiated cells cultured for 3 wk till adherent to the plastic dish to 1% agarose-coated dishes (suspension culture). For the experiments presented in Fig. 4, chondrocytes were dissociated from tibiae of stage 30 chick embryo and cultured directly in suspension condition.

Culture medium was Coon's modified F12 medium (1) lacking ascorbic acid and supplemented with 10% FCS.

Origin and Labeling of cDNA Probes

The cDNA clone pCIII8 ($\alpha 1$ X) was obtained in our laboratory (4); the cDNA clones pCOL3 ($\alpha 1$ I), pCSI ($\alpha 1$ II), and pYN 1731 ($\alpha 2$ IX) were kindly provided by E. Avvedimento (28), M. Sobel (National Institutes of Health, Bethesda, MD; see reference 29), and Y. Ninomiya (Harvard Medical School, Boston, MA) (17), respectively; the genomic clone pXCR7 (Hind III–Hind III fragment of *Xenopus laevis* rDNA) was a gift from F. Amaldi (Universita Tor Vergata, Rome, Italy).

Probes were labeled by the standard nick translation method as described by Maniatis et al. (12). The specific activity of the probes was always $>10^8$ cpm/ μ g of DNA. Whole plasmids were used as probes only when filters were hybridized for the first time. To avoid possible cross-reaction when the filters were washed and reused for hybridization with different probes, only the cDNA inserts were used after excision from the vectors by restriction enzymes and purification on an agarose gel/NA 45 membrane (Schleicher & Schuell, Inc., Keene, NH).

RNA Extraction and Hybridization Analysis

RNA was extracted by guanidinium isothiocyanate/CsCl density gradient centrifugation, denatured, and subjected to electrophoresis through 1% agarose gels containing formaldehyde or glyoxal according to the procedure described by Maniatis et al. (13). Northern blots were performed by capillarity on a nylon membrane (Hybond; Amersham Corp., Arlington Heights, IL). RNA slot blots were performed with a manifold apparatus (Schleicher & Schuell, Inc.) after denaturation at 65°C for 15 min in 4 \times SSC (1 \times SSC solution is 0.15 M sodium chloride, 0.015 M sodium citrate) containing 25.9% formaldehyde, and chilling on ice for 15 min. (24). Denatured RNA was spotted at concentrations of \sim 20, 5, and 1.25 μ g on a Hybond nylon membrane.

Binding of RNA to membrane and hybridization were carried out according to the directions of the membrane manufacturer with the following modifications: hybridization and washing temperature was 54°C; filters were washed four times in 1 \times SSPE (20 \times SSPE solution is 3.6 M NaCl, 0.2 M Na phosphate buffer, pH 7.7, 0.02 M Na₂ EDTA) containing 0.2% SDS for 10 min and then twice in 2 \times SSPE, 0.2% SDS for 30 min to avoid cross-hybridization (6). In addition, when the probe for $\alpha 1$ (II) was used, the formamide concentration was raised from 50 to 60% vol/vol to increase hybridization specificity. When the filters were reused with different probes they were washed, hybridized for 16 h at 74°C in 5 mM Tris-HCl, pH 8.0, 2 mM Na₂ EDTA, 1 \times Denhardt's solution (100 \times solution is 2% wt/vol BSA, 2% wt/vol ficoll 70, 2% wt/vol polyvinylpyrrolidone), and autoradiographed to ensure that no probe remained on the filters. Filters were autoradiographed wet for different times (3–72 h) at -80°C using intensifying screens. Different exposures were scanned at 590 nm with a spectrophotometer (DU8; Beckman Instruments Inc., Palo Alto, CA).

In Vitro Run-off Transcription Assay

Cells were collected, washed once in PBS (pH 7.2), and resuspended at a concentration of 5 \times 10⁶ cells/ml in buffer A (10 mM Tris-HCl, pH 7.6, 40% glycerol, 10 mM MgCl₂, 10 mM NaCl) containing 0.1% Triton X-100, and precooled at -20°C . Cells were then homogenized with 35 strokes of a tightly fitted Dounce homogenizer. Nuclei were pelleted by centrifugation

at 4,000 g for 10 min at -15°C , washed twice in the same buffer without Triton, purified by centrifugation at 150,000 g for 65 min at 4°C through a 1.8-M sucrose cushion in 10 mM Tris-HCl, pH 7.6, and 10 mM MgCl₂, and stored in buffer A at -80°C at a concentration of 1–3 \times 10⁸/ml. Transcription assays were performed according to the method of Clayton and Darnell (5) with the following modifications: nuclei were adjusted to 15–25 absorbance units at 260 nm (determined in 1% SDS), and the reaction medium contained a 1-mM concentration each of ATP, GTP, and CTP, 0.5 U/ml of RNase inhibitor (Boehringer Mannheim), and 125 μ Ci [α -³²P]UTP (3,000 Ci/mmol; Amersham, Corp.). When assaying RNA polymerase II activity, nuclei were preincubated on ice for 10 min in the same buffer supplemented with 1 μ g/ml alpha-amanitin. Transcription incubation was carried out for 30 min at 30°C, the time interval that, in preliminary experiments, allowed an optimal incorporation of [³²P]UTP to occur at that temperature (data not shown). The reaction was terminated by digestion with 100 μ g/ml concentrations each of DNase I and proteinase K in the presence of 1 mM CaCl₂ (25). Yeast tRNA was added to a final concentration of 25 μ g/ml and RNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of ethanol. RNA hybridization was carried out in DNA excess conditions (2 μ g per assay) as described by McKnight and Palmiter (14) with the collagen cDNA inserts excised from the plasmids described above and immobilized on nitrocellulose filters. The length of these cDNA restriction fragments were: Pst I–Pst I, 530 bp for pCIII8; Hind III–Hind III, 800 bp for pCOL3; Pst I–Pst I, 780 bp for pCSI; and Hind III–Rsa I, 1,600 bp for pYN1731. In each assay, nonspecific hybridization was estimated in parallel with a filter devoid of cDNA. After a 3-d hybridization, the filters were washed and digested with RNases A and T₁ (1 μ g/ml and 10 U/ml, respectively) (5). The [³²P]RNAs were eluted (14) and the radioactivity was quantitated in Pico-Fluor 40 (Packard Instrument Co., Downer's Grove, IL). The relative rate of a gene transcription for each type of collagen is expressed as parts per million (ppm), which is the ratio between counts per minute specifically hybridized to the corresponding cDNA and counts per minute \times 10⁻⁶ in total transcription.

Results

Collagen mRNA Levels during In Vitro Differentiation of Chick Embryo Chondrocytes

To avoid uncontrolled loss during fractionation, total RNA was used for analysis. Total RNA was extracted from dedifferentiated chondrocytes grown for 3 wk as adherent cells on tissue culture plastic dishes and at different time intervals after their transfer to suspension culture on agarose-coated dishes. Aliquots of the extracts were analyzed both by Northern and slot blots with probes for the $\alpha 1$ chains of types I, II, and X collagens and the $\alpha 2$ chain of type IX collagen (Figs. 1 and 2). To minimize experimental errors, each hybridized filter was exposed for different lengths of time and only exposures in the proportional range of the autoradiographic film darkening were considered for densitometry scanning analysis. The amount of RNA was normalized with respect to the amount of ribosomal RNA as detected by hybridization with the specific probe pXCR7.

The results indicate that immediately after the transfer of the cells to suspension culture, the level of type I collagen mRNA rapidly decreased to, by the fourth week, a value equivalent to 10% of that determined for the dedifferentiated cells. On the contrary, within the first week, the amount of types II and IX collagen mRNAs dramatically increased and reached \sim 80 and 90%, respectively, of the maximal levels observed at the second week. At later times of culture the amount of types II and IX collagen mRNAs decreased. A different pattern was observed for type X collagen mRNA. This mRNA accumulated more progressively throughout culture, and at the end of the first week its concentration was only 40% of the maximum observed at the fourth week.

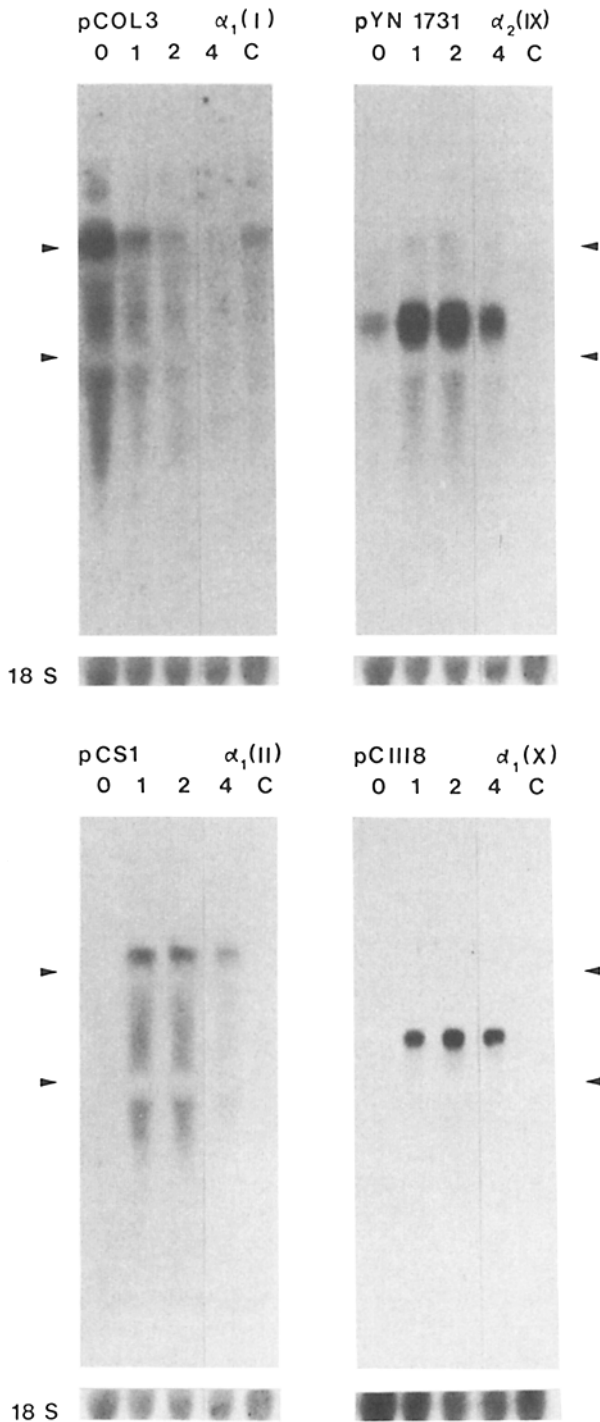


Figure 1. Northern blots of total RNAs extracted from chondrocytes grown for 3 wk on plastic dishes (lanes 0) and from the same cells transferred and cultured on agarose-coated dishes for 1, 2, 3, or 4 wk (lanes 1-4). Control RNA (lanes C) was from chicken liver. On each slot $\sim 30 \mu\text{g}$ of RNA were loaded. Probes used are indicated at top of each panel. Arrows refer to the position of ribosomal RNAs. At the bottom of each panel the 18S rRNA region of the same filter after rehybridization with the pXCR7 probe for rRNA is shown.

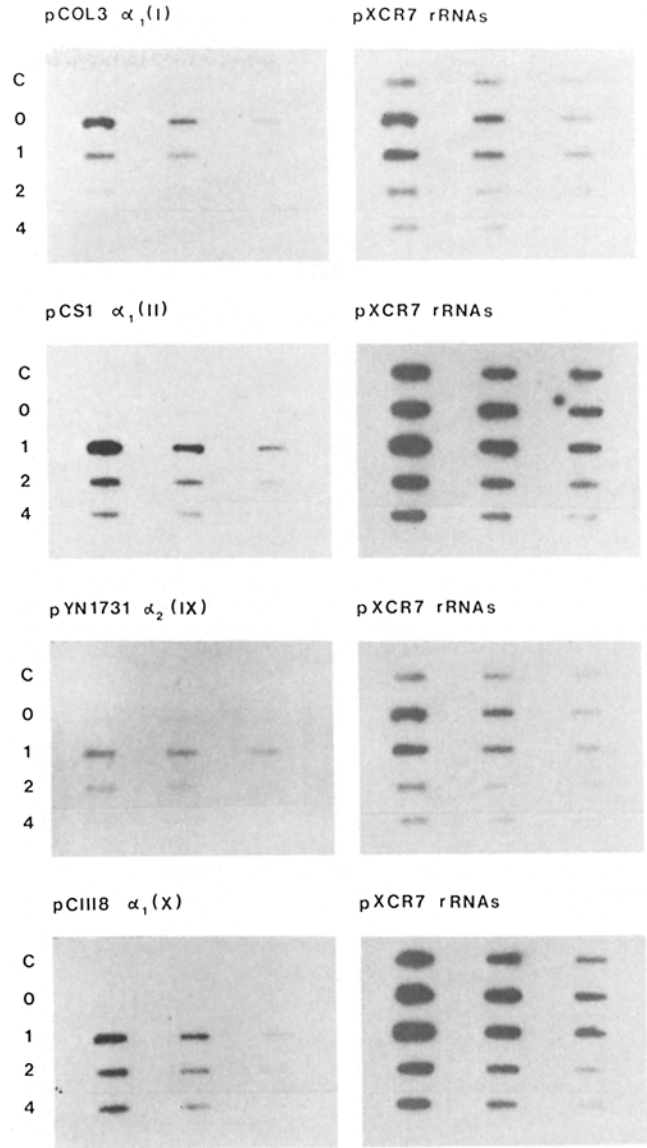


Figure 2. Slot blots of total RNAs extracted from chondrocytes grown for 3 wk on plastic dishes (lanes 0) and from the same cells transferred and cultured on agarose-coated dishes for 1, 2, and 4 wk (lanes 1, 2, and 4). Control RNA (lanes C) was from human lymphoid T cell line. Probes used are indicated on top of each panel. Amount of loaded RNA was ~ 20 , 5, and $1.25 \mu\text{g}$. The filters were hybridized first with the collagen-specific probe and then with the rRNA probe. For types I and IX collagen probes the same filter was used.

It must be noted that low but detectable levels of cartilage-specific mRNAs were observed in adherent cells.

Transcriptional Activity of Collagen Genes in Differentiating Chondrocytes

To investigate whether modifications in the rate of transcription of the collagen genes could account for the changes in mRNA levels described above, we performed, in an independent experiment, in vitro run-off transcription assays on nuclei isolated from cells cultured either adherent to the dishes or grown in suspension for 1-4 wk. Control assays

Table I. Relative Rate of Transcription of the Genes Encoding Types I, II, IX, and X Collagens during Chondrocyte Differentiation In Vitro

Culture conditions	Total transcription <i>cpm</i> × 10 ⁻⁶	Collagens			
		Type I <i>ppm</i>	Type II <i>ppm</i>	Type IX <i>ppm</i>	Type X <i>ppm</i>
Dedifferentiated (adherent)	24.4	207	2	5	11
Dedifferentiated + α-amanitin	11.7	45	0.95	1.6	2.3
1-wk Suspension	18.7	127	35	74	218
2-wk Suspension	20.9	98	36	61	419
2-wk Suspension + α-amanitin	12.7	18	12	14	88
3-wk Suspension	24.0	57	30	66	643
4-wk Suspension	22.7	19	15	58	438

Dedifferentiated chondrocytes cultured as adherent cells for 3 wk were transferred to suspension culture for 1-4 wk. The rate of transcription of the genes encoding collagen types I, II, IX, and X was measured by the level of incorporation of [³²P]UTP into nascent RNA transcripts in isolated nuclei and is expressed as parts per million (ppm) as described in Materials and Methods. Nonspecific hybridization averaged 100, 11, 19, and 54 cpm for collagen types I, II, IX, and X, respectively.

with alpha-amanitin were also included. Transcription data, expressed as parts per million, are presented in Table I. The transfer of dedifferentiated adherent cells to a suspension culture allowing cellular differentiation resulted in a marked and continuous decrease in the transcriptional activity of the gene encoding collagen type I. In contrast, in the same differentiating cells, the transcription rate of the genes specific for collagen types II, IX, and X was significantly enhanced, the highest increase being that for the gene encoding collagen type X (~60-fold stimulation with respect to dedifferentiated cells). These three genes showed an overall similar pattern of activation, reaching maximal transcription rate between the first and second weeks (collagen types II and IX) or within the third week (collagen type X), and decreasing thereafter to a lower level at the fourth week of suspension culture. Finally, all the changes in gene transcription rate described above were specific since (a) the change of culture conditions (adherent to suspension) did not alter the total nuclear RNA synthesis, as shown by the total transcription ranging from 19-24 × 10⁶ incorporated counts per minute; (b) in the presence of 1 μg/ml of alpha-amanitin,

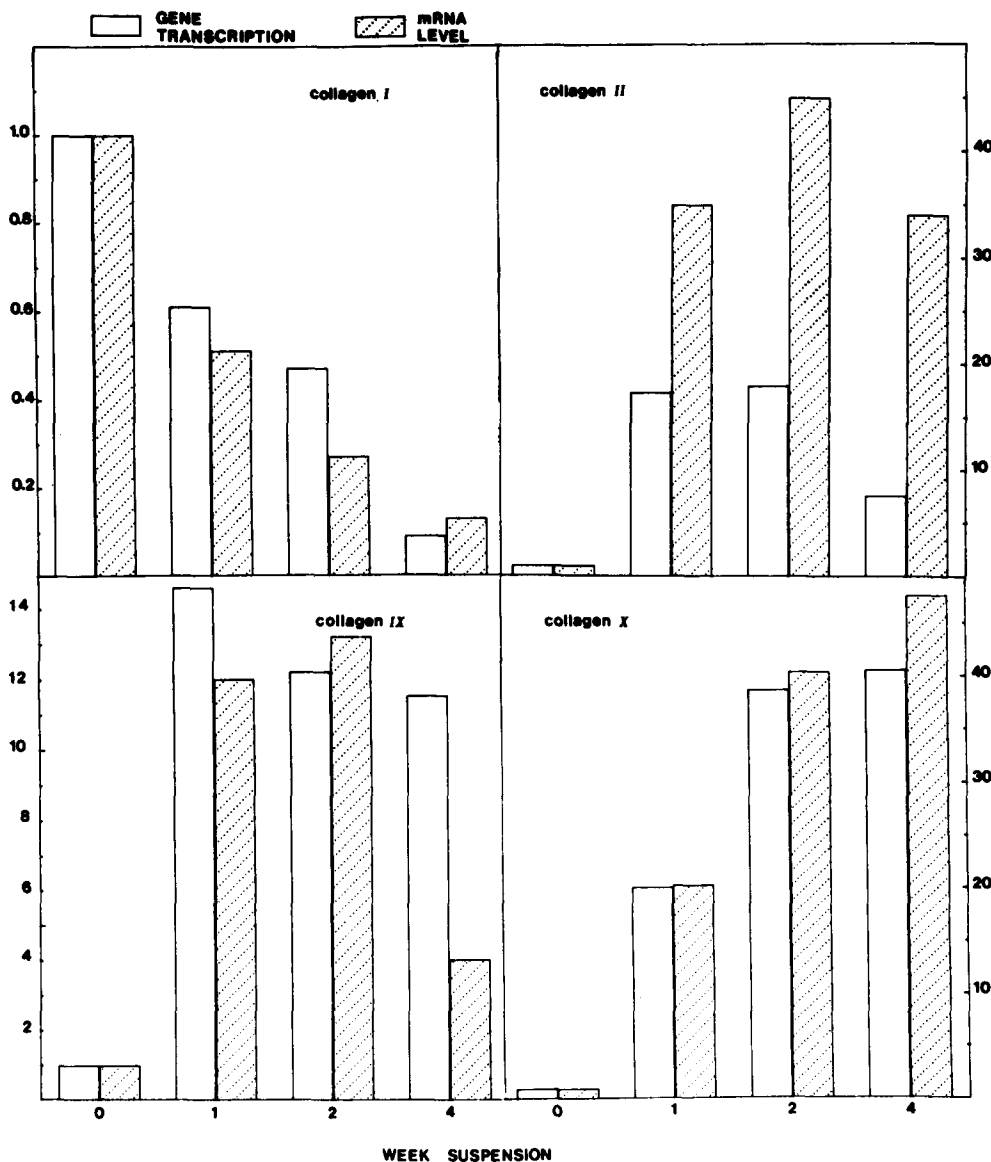


Figure 3. Levels of mRNAs and gene transcription activity for collagen types I, II, IX, and X during chondrocyte differentiation in vitro. Vertical bars represent the ratios between each value of suspension culture and the value determined on the dedifferentiated cells, which was taken as 1.0, for both mRNA level and gene transcription activity. The relative amounts of mRNAs were calculated from densitometric analysis of different exposures of Figs. 1 and 2. Each filter was hybridized with the specific probe pXCR7 and for each slot the value of mRNA was normalized on the basis of rRNA content. The values of gene transcription used are those reported in Table I.

the incorporation of [³²P]UTP into collagen RNA transcripts was inhibited by 75–80%, which indicated that these transcripts resulted from RNA polymerase II activity (12). A comparative summary of the evolution of mRNA levels and transcription activity for the four collagen genes during *in vitro* differentiation is given in Fig. 3. As can be seen, the decrease in the rate of transcription of the gene encoding collagen type I was proportional to the decrease in mRNA level at each time interval. Also for collagen types IX and X, the extent of transcription activation was sufficient to account for the accumulation of the respective mRNAs. In contrast, collagen type II mRNA accumulated in excess as compared with the increase in transcriptional activity of the corresponding gene.

Collagen II and X mRNA Levels at the Late Stage of Differentiation of Chick Embryo Chondrocytes

To further investigate the evolution of types II and X collagen mRNAs at a late stage of differentiation, chondrocytes freshly dissociated from stage 30 chick embryo tibiae were directly cultured in suspension. Total RNA was extracted from the tissue itself as from cells cultured for 2, 7, and 21 d. Fig. 4 shows the levels of these mRNAs, estimated from scanning of the Northern blots. A continuous decrease of the amount of the messenger for collagen type II was observed, whereas the amount of collagen type X increased to reach a peak at 7 d and decreased thereafter.

Discussion

In this paper we report studies on the steady-state levels of collagen mRNAs and on the transcriptional activity of the specific genes during the differentiation *in vitro* of chicken hypertrophic chondrocytes starting from adherent dedifferentiated cells. The data presented elucidate some new aspects of the expression of collagen genes during this process.

Type II collagen mRNA dramatically increases within the first week after the transfer of the cells to suspension and reaches its maximal level in the second week. During limb development and in micromass cultures *in vitro* an increase in the amount of type II collagen mRNA is concomitant with the condensation phase of mesenchymal prechondrogenic cells (10). The condensation phase is considered to be the first major event during chondrogenesis. It is noteworthy that in our culture system adherent dedifferentiated cells undergo an aggregation phase immediately after their transfer to suspension.

The change in the transcriptional activity of type II collagen gene has the same pattern as the one of its messenger RNA, reaching a peak at the second week; however, the higher level of mRNA as compared with the rate of transcription suggests that an additional stabilization of this mRNA could also take place during differentiation. Accumulation of type IX collagen mRNA follows a pattern similar to the one of the mRNA for collagen type II. We observed that the increase in mRNA for collagen type IX is mostly due to transcription activation, although the slight discrepancy between rate of transcription and level of mRNA at the fourth week could indicate an accelerated degradation of the mRNA.

Mueller-Glauser et al. (16) have recently shown that type IX collagen is localized in the intersections of type II collagen fibrils and probably contributes to their stabilization. It

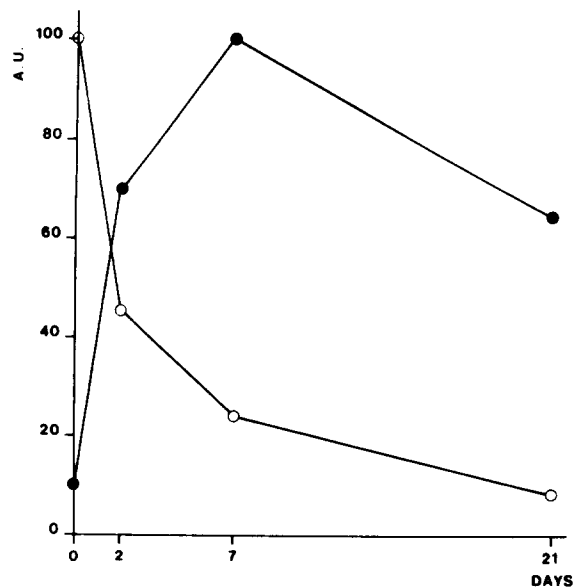


Figure 4. Levels of types II and X mRNAs in chondrocytes differentiated *in vivo* and completing their maturation *in vitro*. Total RNAs were extracted from chondrocytes derived from 29–31 stage chick embryo tibiae (0 d) and from the same cells cultured for 2, 7, and 21 d on agarose-coated dishes. The RNA was analyzed by Northern blots (not shown) and the amount of RNA was estimated from densitometric scannings. Values are expressed in arbitrary units, assuming as 100 the highest value for each RNA. (Solid circles) Type X collagen mRNA; (open circles) type II collagen mRNA.

is tempting to speculate that the simultaneous increase of types II and IX mRNAs reflects the necessity of a correct assembly of extracellular fibrils for the contemporary occurrence of both collagens.

Type X collagen mRNA increase is more continuous and progressive and reaches its maximum at 3–4 wk, when essentially all cultured cells are hypertrophic. At a later stage of cell differentiation a decrease is also observed in type X collagen mRNA (see Fig. 4). This could be due both to an additional specific regulation mechanism of collagen expression or to a senescence phenomena. The accumulation of type X collagen mRNA by differentiating hypertrophic chondrocytes is specifically inhibited by the presence of 180 mM dimethylsulfoxide in the culture medium (15). We can now ask which stimulus induces in chondrocytes the transcription of type X collagen mRNA both *in vivo* and *in vitro*. Micro-environment changes are certainly involved. The culture conditions may play a relevant role since chondrocytes from the permanent cartilaginous caudal zone of chicken sternum, when grown on agarose-coated dishes, also differentiate to hypertrophic chondrocytes synthesizing type X collagen (4, 21).

Our results further indicate that during the *in vitro* differentiation process the level of type I collagen mRNA (relatively high in the adherent dedifferentiated cells) rapidly decreases, though a detectable amount is still present in well differentiated chondrocytes. These results are in agreement with those obtained *in vivo*, where both the switch from synthesis of type I to type II collagen and the presence of low levels of type I collagen mRNA in already differentiated chondrocytes were observed (26). A translational down-regulation of type I collagen during the chondrogenesis pro-

cess has been reported by other investigators (8, 10, 19, 11). In this paper we show that the transcriptional activity of the type I collagen gene decreased with differentiation. Therefore, the regulation of this messenger RNA occurs at the level of transcription. In vivo, low but detectable levels of type II collagen mRNA are present in mesenchymal cells at stage 20/22 of limb development, well before overt cartilage differentiation (11), and it has been suggested that a low level of expression of type II collagen gene represents a molecular marker of the state of determination of progenitor cells (10). In agreement with this finding, our results indicate low but detectable transcription activity and mRNA levels for cartilage-specific collagen in the dedifferentiated cells. It cannot be ruled out, however, that a small number of contaminating differentiated chondrocytes are responsible for the presence of detectable types II, IX, and especially X collagen mRNAs.

After 2 wk of culture the amount of types II and IX collagen mRNAs in chondrocytes decreases. A decrease of type II collagen mRNA has been also reported by Kravis and Upholt (11) in prolonged culture of chondrocytes; nevertheless, given their culture conditions, it is likely that the decrease they observed was due, at least partially, to a dedifferentiation of chondrocytes.

It is important to mention here that we have several lines of evidence suggesting that, in our culture system, the first stage concerned with the high expression of collagen type I as well as the subsequent reversion to the synthesis of cartilage-specific collagens might reflect the in vivo situation. (a) The dedifferentiated cells were obtained by enzymatic digestion of whole tibiae of stage 28/30 chicken embryo. Such cell population used as a starting point of the in vitro culture is heterogeneous in terms of stage of differentiation; nevertheless, all cells assume the fibroblast phenotype and revert to type I collagen synthesis. (b) Cloned cells obtained by limiting dilution of the freshly dissociated 6-d embryo tibia, when grown in anchorage-dependent conditions (fibroblast-like morphology) and transferred thereafter in suspension culture in the constant presence of ascorbic acid, are able to differentiate to form a structure analogous to hypertrophic cartilage (23). (c) We have evidence that cultured prechondrogenic limb bud cells can undergo a sequence of morphological and biochemical events almost superimposable to those described with dedifferentiated cells (Tachetti, C., B. Dozin, and R. Cancedda, manuscript in preparation).

We have also to point out that in our culture system the decrease in the types II and IX collagen mRNAs is concomitant with the achievement of the highest type X collagen mRNA level.

Given all that, we suppose that during chondrocyte differentiation, after the first shift in the collagens synthesized at the time chondrogenesis begins, a second shift occurs between type II (and IX) and type X collagen when chondrocytes become hypertrophic.

In summary, based on the data presented in this paper and on our previous observations on collagen synthesis (3) and kinetic properties of chicken chondrocyte undergoing differentiation (Giaretti, W., G. Moro, R. Quarto, S. Bruno, A. DiVinci, E. Geido, and R. Cancedda, manuscript submitted for publication), we suggest that chicken chondrocyte differentiation proceeds through at least two different steps: (a) a

transition from a stage characterized by a high level of type I collagen mRNA to a stage characterized by a predominance of types II and IX collagen mRNAs and (b) a transition to a stage characterized by the highest level of type X collagen mRNA.

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