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# Alterations of Collagen mRNA Expression During Retinoic Acid Induced Chondrocyte Modulation: Absence of Untranslated α1(I) mRNA in Hyaline Chondrocytes

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**Abstract** Retinoic acid (RA) has been shown to rapidly modulate the collagen expression pattern of chondrocytes in vitro at doses of 1–10 μM Embryonic chicken sternal chondrocytes stop synthesizing the cartilage-specific type II collagen within 2–4 days of RA treatment and turn on the synthesis of types I and III collagen and fibronectin While suppression of type II collagen synthesis and onset of type III collagen and fibronectin synthesis have been shown to be regulated at the transcriptional level, conflicting data are available on a possible post-translational regulation of α1(I) collagen gene expression In this study we demonstrate by comparing a commonly used α1(I) cDNA probe from the 3′ end of the α1(I) mRNA with a newly prepared α1(I) specific cDNA probe from the 5′ end (p1E1) that—in contrast to previous reports—chicken sternal chondrocytes do not contain untranslated α1(I) mRNA which may become translatable after RA treatment By in situ hybridization we show the absence of cytoplasmic α1(I) mRNA from chondrocytes and its presence in the perichondrium of sternal cartilage Perichondral cells might have contaminated sternal chondrocyte preparations, explaining low levels of α1(I) mRNA seen by Northern hybridization and RNase protection assays of chicken sternal cartilage mRNA even with the p1E1 probe We show by Northern hybridization and metabolic labeling with $^3$H-proline followed by SDS-gel electrophoresis that retinoic acid at 3 μM suppresses type II, IX, and X collagen gene expression within 2 days both at the mRNA and protein level and induces the onset of α1(I), α2(I), and α1(III) expression within 3 days No expression of CRABP, the cellular retinoic acid binding protein, was seen in RA-treated or control chondrocytes, indicating that CRABP protein is not involved in the RA-induced modulation of the chondrocytes © 1993 Wiley Liss Inc

**Key words:** CRABP, retinoic acid, collagen, chondrocytes, sternal cartilage

Chondrocytes of hyaline cartilage synthesize a distinct pattern of the collagen types II, IX, and XI which provide the fibril network of fetal cartilage anlagen and adult articular cartilage. In situ the expression pattern of these cartilage-specific collagens is strictly controlled under normal conditions. Alterations in the collagen expression pattern observed in osteoarthritis—for example, the onset of types I, III, or X collagen [Gay et al., 1976; Adam and Deyl, 1983; von der Mark et al., 1992a; Aigner et al., 1992]—can lead to the formation of an abnormal cartilage matrix with reduced stability and endurance [von der Mark et al., 1992b]. Experimental evidence for such phenotypic instabilities of chondrocytes is available from numerous cell culture studies [for overview see von der Mark, 1986]. In monolayer culture hyaline spherical chondrocytes flatten, spread, and assume fibroblastoid cell shape including focal contacts and stress fibers [Holtzer and Abbott, 1968; Marchisio et al., 1984; Brown and Benya, 1988], an event which finally leads to a modulation of the collagen pattern from types II, IX, and XI to types I, III, and V [Mayne et al., 1976a; Muller et al., 1977; von der Mark et al., 1977; Benya and Padilla, 1978]. This modulation can be induced or accelerated by a number of substances such as bromodeoxyuridine [Schiltz et al., 1973; Mayne et al., 1984] embryo extract [Mayne et al., 1976b], fibronectin [West et al., 1979; Pennypacker et al., 1979], and retinoic acid [Benya

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and Padilla, 1986; Horton et al., 1987; Yasui et al., 1986; Oettinger and Pacifici, 1990], although presumably by different mechanisms.

For studies on the molecular mechanism of the regulation of collagen gene expression in chondrocytes, retinoic acid (RA) has received particular interest in the past in light of the DNA-binding capacity and transcription-regulating ability of the retinoic acid receptor [Giguere et al., 1990; Petkovitch et al., 1987] and its physiological role in cartilage and limb formation [Tickle et al., 1982; Summerbell, 1983; Brockes, 1989]. The data published so far on the effect of RA on the collagen gene expression in chondrocytes, however, are not entirely consistent. There is agreement in the finding that RA at doses between 0.1 and 3 μM completely suppresses the expression of type II collagen and aggrecan (chondroitinsulfate-proteoglycan) within 1–2 days [Benya and Padilla, 1986; Yasui et al., 1986; Horton et al., 1987] and turns on the synthesis of type III collagen and fibronectin [Benya and Padilla, 1986; Horton et al., 1987]. Both suppression of type II collagen and onset of type III collagen expression by RA are regulated at the transcriptional level [Horton et al., 1987]. Conflicting data, however, exist on the expression of the α1 and α2 subunits of type I collagen in chondrocytes with and without the influence of RA. While the induction of α1(I) synthesis by RA has been demonstrated unequivocally at protein level [Benya and Padilla, 1986; Yasui et al., 1986], there has been some confusion about the identification of α1(I) mRNA in chondrocytes with a cDNA probe derived from the 3′ end of the COL1A1 gene with a high degree of homology to the COL2A1 gene in the corresponding region. Reports on significant levels of untranslated α1(I) mRNA in freshly isolated chicken sternal chondrocytes [Duchene et al., 1982; Focht and Adams, 1984; Askew et al., 1991], which had suggested post-transcriptional control of α1(I) mRNA expression and a transition from an untranslatable to a translatable form of α1(I) mRNA during chondrocyte modulation, were questioned in other studies showing the absence of α1(I) mRNA in the sternal chondrocytes [Gerstenfeld et al., 1989] but its presence in vertebral chondrocytes. Similar, significant levels of untranslated α2(I) mRNA, but no protein, were found in freshly isolated fetal chick chondrocytes [Saxe et al., 1985], with considerable induction of α2(I) mRNA and translation into collagen α2(I) chains after RA treatment [Yasui et al., 1986; Oettinger and Pacifici, 1990]. In contrast, no α2(I) chains were found in rabbit articular chondrocytes after RA treatment [Benya and Padilla, 1986].

In this study we have made attempts to clarify the issue about the presence of untranslatable α1(I) mRNA in chicken chondrocytes and to investigate whether onset of type I collagen expression in chondrocytes is regulated at the translational or transcriptional level. This was achieved by preparing a new α1(I) specific cDNA probe from the 5′ end of the COL1A1 gene and excluding crosshybridization with the COL2A1 gene. Furthermore, we show that the retinoic acid effect does not involve CRABP, the cellular retinoic acid binding protein [Maden et al., 1988; Dolle et al., 1989, 1990].

## MATERIALS AND METHODS

### Sternal Chondrocyte Cell Culture

Sternal chondrocytes were prepared from 17-day-old chicken embryos or from 1-day hatched chickens as previously described [Dessau et al., 1978] with the following modifications: sterna were treated with 0.2% trypsin for 30 min at 37°C, followed by two washes with phosphate buffered saline (PBS) to remove adhering perichondrium. Sterna were digested by incubation with 0.1% collagenase for 8 h in Ham's F12 medium plus 10% fetal calf serum (FCS). The released chondrocytes were harvested by centrifugation and plated onto 100 mm cell culture dishes at a density of approximately $5 \times 10^6$ cells/dish. Cells were cultured in F12 medium supplemented with 10% FCS, 100 mg/l ascorbic acid, 100 U/l penicillin, and 100 mg/l streptomycin at 37°C in a humified atmosphere containing 5% $CO_2$. After 24 h in monolayer culture, retinoic acid (Serva, Heidelberg, Germany) at a concentration of 3 μM (1 μg/ml) was added to the medium to induce dedifferentiation of the chondrocytes.

### Metabolic Labeling of Cells

RA treated and untreated chondrocytes were labeled for 4 h with 20 μCi $^3$H-proline (Amersham) in serum free Dulbecco's Modified Eagle's Medium (DMEM). Cells were scraped off the dishes, washed three times with PBS, and lysed in 500 μl protein sample buffer according to King and Laemmli [1971]. Samples containing 300,000 cpm labeled cellular proteins were separated by SDS-gel electrophoresis using

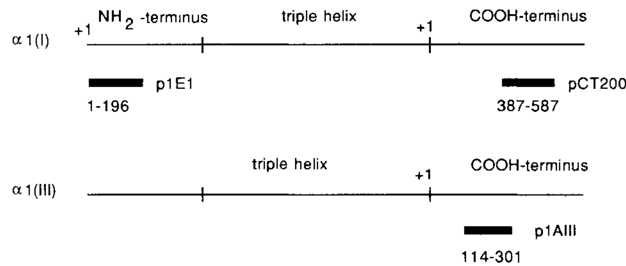


**Fig. 1.** Position of p1E1 and the pCOL3 derived clone pCT200 in the α1(I) mRNA and of p1AIII in the α1(III) mRNA.

5–12% polyacrylamide linear gradient gels. Alternatively, the newly synthesized procollagens were digested with pepsin at 4°C and analysed by SDS-gel electrophoresis. The gels were fixed in 10% acetic acid and 20% methanol for 30 min and soaked for 30 min in a commercial amplifier (Amersham) for fluorography. Gels were dried and exposed to Kodak X-OMAT™ X-ray films.

### RNA Extraction

Control and RA treated chondrocytes were scraped from the cell culture plates, harvested by centrifugation, and lysed in 4 M guanidinium/SCN, 25 mM Na-Citrate, 0.5% Na-Sarcosyl, and 0.7% β-mercaptoethanol. Total cellular RNA was isolated by CsCl centrifugation according to the method of Chirgwin et al. [1979].

### cDNA Probes

For detection of α1(II), α1(IX), α1(X), and α2(I) mRNAs, cDNA fragments derived from the plasmids pCs1 [Young et al., 1984], pYN 1738 [Ninomiya and Olsen, 1984], SPLX [LuValle et al., 1988], and pColl [Sobel et al., 1978], respectively, were used as probes. For detection of mRNAs for α1(I) initially the pCol3 clone derived from the 3′ end of the α1(I) mRNA was used [Yamamoto et al., 1980]. Another cDNA clone derived from the 5′ end of the α1(I) cDNA, as well as probes for α1(III) and the CRABP protein, were generated by PCR after reverse transcription of the respective mRNAs from total cellular RNA; their identity was verified by sequencing. All of the upstream primers used for the PCRs were synthesized with an Eco RI restriction site close to the end, whereas the downstream primers were synthesized with a Bam HI restriction site. Amplified cDNA fragments were cloned unidirectionally into pGem4 after digestion with the restriction endonucleases Eco RI and Bam HI. The position of the resulting plasmids p1AIII, p1E1, pCT200 and pCRABP296 is depicted in Figure 1: p1AIII contains a 186 bp cDNA fragment of the 3′ region of the α1(III) mRNA. A cDNA fragment representing the first exon of α1(I) mRNA was cloned into the p1E1 plasmid. Plasmid pCT200 contains a cDNA fragment covering a 200 bp region of the 3′ end of the α1(I) mRNA. The plasmids p1E1 and pCT200 were cloned for transcription of radiolabeled antisense RNA probes into the pGemZ vector (Promega). The cDNA fragment of pCRABP296 was amplified from total cellular RNA of 4-day-old chicken embryos and consists of 296 bp deduced from a recently published cDNA clone [Vaessen et al., 1990].

### Northern Blot Analysis

RNA was electrophoresed in 20 cm, 1% agarose gels containing 6% formaldehyde and 1 × MOPS buffer (1 × MOPS: 20 mM morpholinopropane sulfonic acid, 5 mM Na-acetate, 1 mM EDTA). Transfer of RNA onto Hybond-N nylon membranes (Amersham) was carried out by capillary blotting with 20 × SSC (1 × SSC: 0.15 M NaCl, 0.015 Na-Citrate). After transfer, the membranes were dried for 10 min at 80°C and RNAs were fixed by UV irradiation of 264 nm for 5 min. For analysis of RNAs, cDNA probes were labeled with $^{32}$P-dATP or dCTP by random priming and hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution [Ausubel et al., 1987], 0.5% SDS, and 100 mg/ml denatured herring sperm DNA at 42°C for 16 h. After hybridization, filters were washed twice in 2 × SSC for 5 min at room temperature and twice in 2 × SSC/0.1% SDS for 30 min each at 65°C. The washed filters were exposed to Kodak X-OMAT™ X-ray films.

### RNase Protection of RNA/RNA Hybrids

Labeled antisense RNA to α1(I) mRNA was prepared by digestion of p1E1 and pCT200 with Eco RI, followed by in vitro transcription with

T7 RNA polymerase. For radioactive labeling, $^{35}$S-UTP was added to the reaction mixture. Labeled RNA was resuspended in 100 μl hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA). Ten micrograms of total RNA, to be assayed, was ethanol precipitated and resuspended in 30 Ìl hybridization buffer containing 5 × $10^5$ cpm of the probe RNA. The samples were incubated for 5 min at 85°C, followed by 16 h at 42°C. After addition of 350 μl ribonuclease digestion buffer (10 mM Tris/Cl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 40 mg/ml RNase A, 2 mg/ml RNase T1), the samples were incubated for 30 min at 37°C. The reaction was stopped by adjustment to 0.5% SDS and digestion with proteinase K (125 μg/ml) for 15 min at 37°C. The samples were phenol extracted, ethanol precipitated, and resuspended in 10 μl RNA loading buffer (80% formamide, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). The protected fragments were heated to 85°C and analyzed on a 8 M urea, 6% polyacrylamide gel.

### In Situ Hybridization

In situ hybridization of sternal cartilage was performed according to Aigner et al. [1992]. Cartilage specimens were fixed in 4% paraformaldehyde, dehydrated in a series of ethanol washes, and embedded in paraffin wax. Sections were mounted on slides pretreated with 3-triethoxysilylpropylamine and paraffin was subsequently removed by treatment with xylene. The samples were rehydrated, treated with proteinase K (20 μg/ml), fixed again with 4% paraformaldehyde, and acetylated in 0.25% acetic acid anhydride (in 0.1 M triethanolamine, pH 8.0). After another series of dehydration, the samples were hybridized at 42°C for 12–16 h with antisense riboprobe with a specific activity of 20,000–80,000 cpm/μl in hybridization buffer (50% formamide, 10% dextransulfate, 10 mM DTT, 1 mg/ml tRNA, 300 mM NaCl, 10 mM Tris/Cl, pH 7.4, 10 mM $Na_2PO_4$, pH 6.4, 5 mM EDTA, 0.02% ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% BSA). Washing was performed in 2 × SSC/0.5% β-mercaptoethanol (40°C) and 0.5 × SSC/0.5% β-mercaptoethanol (40°C) for 15 min each. The background was decreased by incubation with RNase A (20 μg/ml) and RNase T1 (50 U/ml) in 0.5 M NaCl, 10 mM Tris/Cl, pH 7.5, plus 5 mM EDTA at 40°C for 30 min. Stringent washing with 2 × SSC/0.5% β-mercaptoethanol and 50% formamide at 45°C was followed by 2 washes in 2 × SSC for 15 min each, and an overnight wash in 0.1 × SSC at room temperature. The sections were dehydrated in a series of ethanol, subsequently dried, coated with autoradiography emulsion diluted 1:1 in water (Kodak Type NTB2; Eastman Kodak, Rochester, NY), and exposed for 3 days at 4°C. Slides were developed (Dektol developer and Unifix fixing reagent; Kodak) and counterstained with Giemsa dye.

## RESULTS

### Effect of Retinoic Acid on Morphology and Collagen Synthesis of Chicken Chondrocytes

Sternal chicken chondrocytes were cultured in monolayer for 1 day. Retinoic acid at a concentration of 3 μM was added after 24 h to induce dedifferentiation. More than 50% of the cells flattened and assumed fibroblastoid morphology within 2 days, which is in agreement with similar studies by Horton et al. [1987] and Benya and Padilla [1986]. After two further days of RA treatment the entire chondrocyte cell population became fibroblast-like.

Changes in types of the collagen synthesized by chondrocytes under the influence of RA were analyzed by metabolic labeling of proteins with L-(2,3)-$^3$H-proline. The newly synthesized procollagens were analyzed by SDS-gel electrophoresis using 5–12% polyacrylamide linear gradient gels. As shown in Figure 2, the synthesis of type II procollagen stopped after 48 h of RA treatment, confirming previously published results [Yasui et al., 1986; Horton et al., 1987].

One day after type II procollagen synthesis was turned off, the cells began to synthesize type I procollagen. On day 5 of RA treatment type I collagen synthesis reached a maximal level (Fig. 2).

### Detection of Collagen mRNAs

Northern blots were performed to correlate the synthesis of type I and type II collagens with the steady state level of their mRNAs and to investigate the steady state levels of other cartilage collagen mRNAs during RA induced dedifferentiation. Total cellular RNA from freshly isolated (control) chondrocytes and from chondrocytes treated for up to 8 days with RA was tested by hybridization with different radiolabeled cDNA probes specific for α1(II), α1(IX),

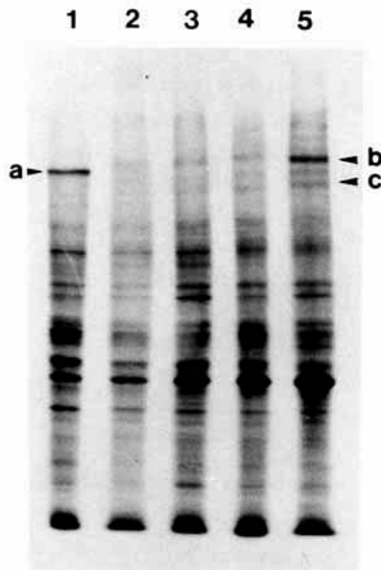


Fig. 2. SDS-polyacrylamide gel electrophoresis of procollagens synthesized with L-(2,3)-$^3$H-proline during RA treatment of chondrocytes Embryonic chick sternal chondrocytes were labeled as described in Materials and Methods Total cellular proteins from control chondrocytes (**lane 1**) and from chondrocytes treated for 2 days (**lane 2**), 3 days (**lane 3**), 4 days (**lane 4**), and 5 days (**lane 5**) with RA at a concentration of 3 μM were reduced with DTT and electrophoresed on a 5–12% SDS-polyacrylamide gradient gel Labeled proteins (300,000 cpm) were loaded to each lane and detected by fluorography Detected procollagens are indicated by letters a, pro α1(II), b, pro α1(I), c, pro α2(I) Note the absence of pro α1(I) in lane 1

Fig. 4. Steady state levels of α1(II) mRNA in untreated control chondrocytes after 2 (**lane 1**), 4 (**lane 2**), 6 (**lane 3**), and 8 (**lane 4**) days in monolayer culture Each lane contains 10 μg of total RNA which was hybridized to the pCS1 probe as in Fig 3

α1(X), α1(I), α2(I), and α1(III) mRNAs. Untreated control chondrocytes contained large amounts of α1(II), α1(IX), and α1(X) mRNAs. After 2 days of growth with RA α1(II) mRNA was only barely detectable (Fig. 3), while in untreated control cultures a significant decline of α1(II) mRNA levels was only visible after 6 days (Fig. 4). The α1(IX) mRNA and α1(X) mRNA disappeared completely after 4–6 days of retinoic treatment.

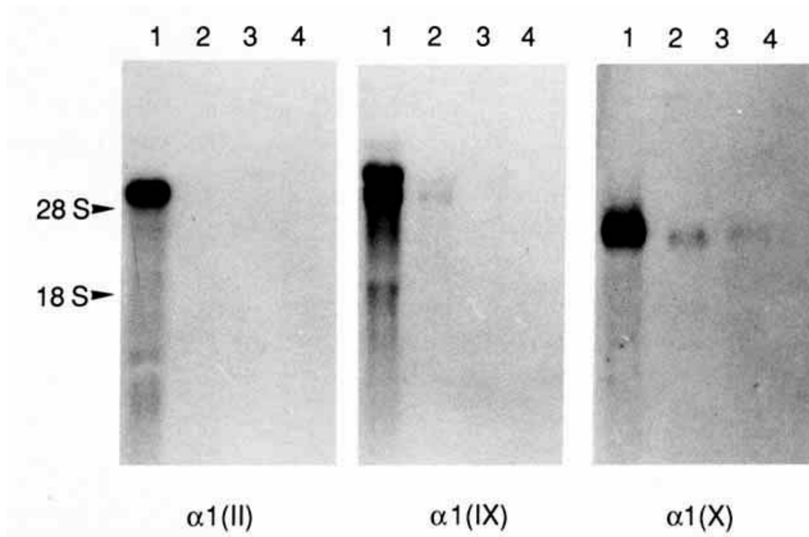


Fig. 3. Steady state levels of cartilaginous collagen mRNAs during RA treatment For Northern blot analysis equal quantities of total RNA (10 μg) from freshly isolated control chondrocytes (**lane 1**) and from chondrocytes treated for 2, 4, and 6 days with RA (**lane 2–4**) were hybridized to cDNA probes specific for α1(II) (pCs1), α1(IX) (pYN1738), and α1(X) mRNA (SPLX) Detected mRNAs are labeled at the bottom of each panel

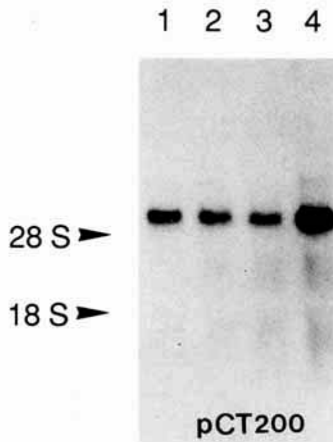


Fig. 5. Steady state levels of α1(I) mRNA in chondrocytes after treatment for 2 (**lane 2**), 4 (**lane 3**), and 6 days (**lane 4**) with retinoic acid **Lane 1:** Freshly isolated chondrocytes RNA 10 μg was hybridized to the pCT200 probe derived from the 3′ end of α1(I) mRNA

Concomitant with the disappearance of chondrocyte specific collagen mRNAs, the levels of α1(I), α2(I), and α1(III) mRNAs increased (Figs. 5, 6). Strong signals were obtained in 1-day control chondrocytes with the pCT 200 probe derived from the 3′ end of COL1A1 (Fig. 5), in accordance with previous reports [Duchene et al., 1982; Focht and Adams, 1984]. Considerably lower but significant levels of α1(I) mRNA were detected with the p1E1 probe (Fig. 6). Also significant levels of α2(I) mRNA were found in freshly isolated sternal cells at day 0 (Fig. 6). Levels of α1(I) and α2(I) mRNA started to increase after 2 days of RA treatment. In contrast, no α1(III) mRNA was seen in control chondrocytes, but became detectable at the time when the type I collagen mRNAs started to increase (Fig. 6). The appearance of α1(III) mRNA in chondrocytes grown for 2 days with RA is in agreement with the findings of others [Horton et al., 1987]. Maximal levels of the collagen mRNAs characteristic for dedifferentiated chondrocytes were reached after 4 days treatment with RA (Figs. 6, 7).

## RNase Protection Assays

The amount of α1(I) mRNA observed after Northern hybridization in 1-day control chondrocytes depended on the cDNA probes used. When hybridization was done with pCT200, a pCol3 derived clone with a cDNA-insertion coding for a portion of the globular domain at the carboxyterminal end of α1(I) mRNA, rather high levels of α1(I) mRNA seemed to be present in control chondrocytes compared to the signals detected with p1E1 derived from the first exon at the 5′ end of the α1(I) mRNA. Sequence comparisons between α1(I) and α1(II) mRNA, however, strongly suggested cross-hybridization of pCT200 with α1(II) mRNA. In the pCT200 clone a stretch of 88 bp showed 90% identity to the corresponding sequence of the COL2A1 gene, while there is virtually no homology in the first exon of the two genes.

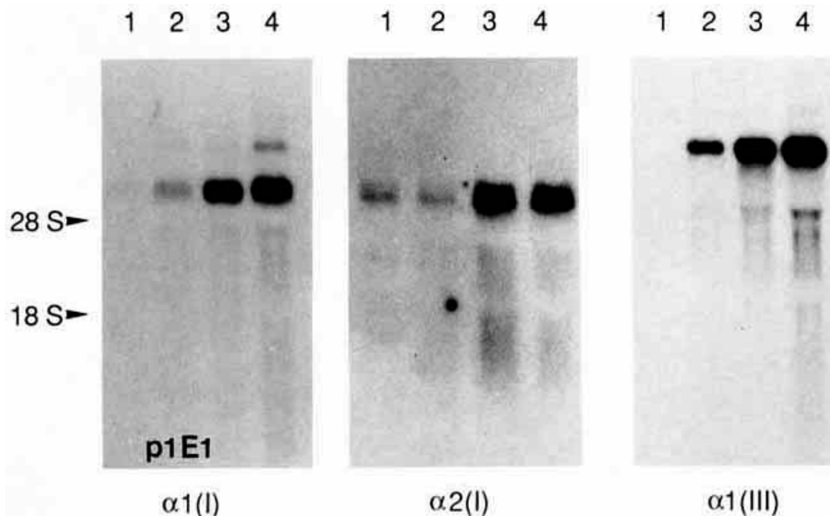


Fig. 6. Steady state levels of collagen mRNAs induced during RA treatment Northern blots shown in Fig 3 were stripped and hybridized to $^{32}P$-labeled cDNA probes specific for α1(I) (p1E1), α2(I) (pCol1), and α1(III) mRNA (p1AIII) Detected mRNAs are indicated at the bottom of each panel **Lane 1:** Control chondrocytes **Lanes 2–4:** Chondrocytes treated for 2, 4, and 6 days, respectively, with RA

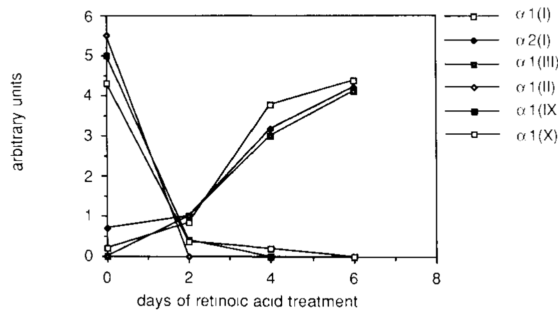


**Fig. 7.** Densitometric analysis of Northern blots shown in Figs 3 and 6 showing relative amounts of mRNAs for indicated collagens during treatment with RA

To verify the results derived from Northern blot analysis, RNase protection assays were performed with two different radiolabeled antisense RNA probes transcribed from p1E1 and pCT200. Both antisense RNA probes were hybridized to tRNA, to total RNA from control chondrocytes, and to RNA from chondrocytes treated for 6 days with RA. In control chondrocytes low levels of α1(I) mRNA fragments were protected from RNase digestion by both antisense probes (Fig. 8, lane 2, upper arrowheads). Both probes gave no signal after hybridization to tRNA, showing absence of unspecific background (Fig. 8, lane 1). Protected α1(I) mRNA fragments were highly increased when the probes were hybridized to total RNA from dedifferentiated chondrocytes (Fig. 8, lane 3), confirming the results from Northern blots. As expected, however, antisense RNA transcribed from pCT200, but not from p1E1, protected also considerable amounts of additional smaller fragments when hybridized to total RNA from control chondrocytes, the most prominent with a length of 32 bp (Fig. 8B, lane 2), indicating partial cross-hybridization to α1(II) mRNA.

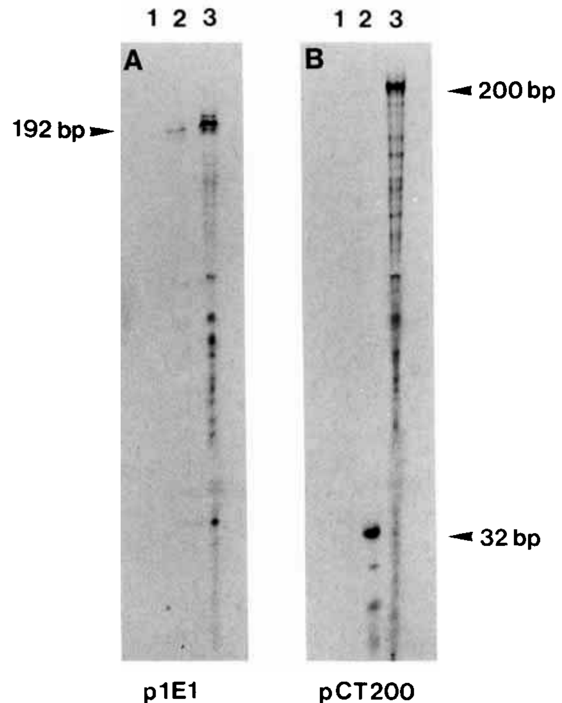


**Fig. 8.** RNase protection analysis of collagen mRNAs in control chondrocytes and chondrocytes treated for 6 days with RA **A:** RNase protection assay with $^{35}$S-labeled antisense RNA transcribed from p1E1 Antisense RNA was hybridized to 30 μg tRNA **(lane 1)**, and 10 μg total RNA from each control **(lane 2)** and RA **(lane 3)** treated cells Fragments protected from RNase digestion were analyzed on a 6% denaturing urea polyacrylamide gel Arrowhead 192 bp fragment of α1(I) mRNA protected by p1E1 **B:** RNase protection assay with antisense RNA transcribed from pCT200 Samples were loaded as described in legend to Fig 6 The lower arrowhead shows a 32 bp fragment of α1(II) mRNA protected by pCT200 The upper arrow marks the protected 200 bp fragment of α1(I) mRNA In addition to high levels of protected α1(I) mRNA fragments in RNA from RA treated chondrocytes, low but significant amounts were also detected by both antisense RNA probes in untreated control chondrocytes

## In Situ Hybridization

Since the RNase protection assay indicated the presence of small but not neglegible amounts of α1(I) mRNA in the sternal chondrocyte preparation, in situ hybridization analysis of this tissue was performed for localization of the α1(I) collagen gene transcripts. Antisense RNA transcribed from p1E1 and pCT200 were used as probes. After hybridization to sections of sterna of 17-day-old embryos positive signals were seen with both probes in cells of the perichondrium (Fig. 9), and in osteoblasts of the chondro-osseus junction of 1-day-old chicken sterna (not shown). In chondrocytes of the resting caudal or hypertrophic cephalic region of 17-day fetal sterna, no α1(I) mRNA transcripts could be detected with the p1E1 probe, while considerable signals were obtained with antisense RNA transcribed from pCT200, indicating crossreaction with α1(II) mRNA (Fig. 7). Therefore, we conclude that the residual α1(I) mRNA seen by RNase protection is not transcribed in chondrocytes, but results from perichondrial cells remaining in the sternal chondrocyte preparation even after removal of the perichondrium.

## Detection of CRABP mRNA

CRABP is a candidate regulator of endogenous RA concentration, and thus might be re-

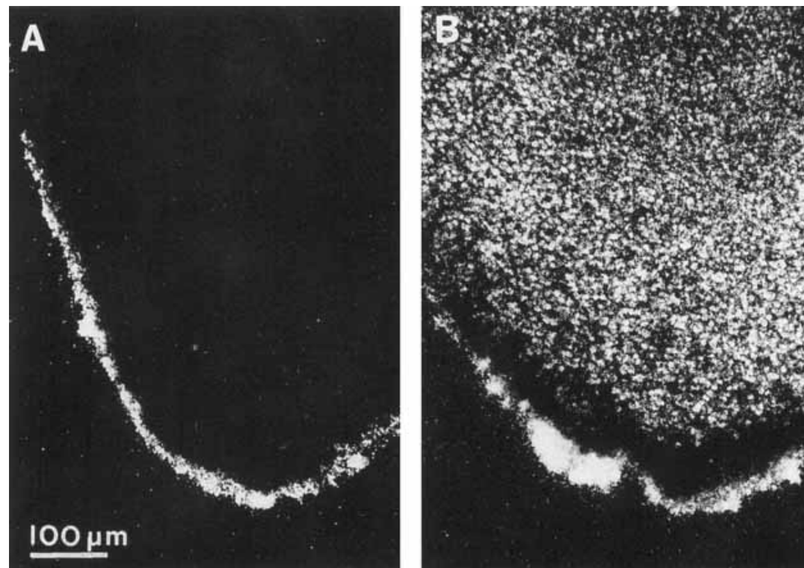


**Fig. 9.** Localization of α1(I) mRNA by in situ hybridization in 17-day embryonic chicken sterna. Panels A and B show sections through the caudal part of the sternum. For in situ hybridization $^{35}$S-labeled antisense RNA transcribed from p1E1 (**A**) and pCT200 (**B**) were used as probe. In contrast to the dark field micrographs in A where specific signals of α1(I) mRNA were detected only in the perichondrium, the micrograph in B shows cross-hybridization between α1(I) antisense RNA and α1(II) mRNA in chondrocytes.

sponsible for the regulation of gene transcription via retinoic acid receptors. To investigate whether this protein plays a role in dedifferentiation of chondrocytes driven by RA, attempts were made to detect CRABP mRNA in control and RA modulated chondrocytes. Radiolabeled cDNA fragments derived from pCRABP296 were hybridized to Northern blots of various RNAs. As positive control for CRABP expression, 20 µg of total cellular RNA from 4-day-old chicken embryos were blotted onto nylon membranes. For detection of CRABP mRNA transcripts in control chondrocytes or in chondrocytes grown for 6 days with RA, 5 µg poly $A^+$ RNA of each was blotted. After hybridization and following exposure for 1 week, only a signal from the RNA derived from 4-day-old chicken embryos was seen (Fig. 10). Neither in control chondrocytes nor in RA treated chondrocytes was CRABP mRNA detected, even when the filters were exposed for 2 weeks.

## DISCUSSION

As a powerful tissue culture model for studies on the regulation of collagen gene expression, the retinoic acid induced modulation of chondrocytes has been the subject of a number of interesting but in part also conflicting studies. Consistent with the reports by Benya and Padilla [1986], Yasui et al. [1986], and Horton et al. [1987], we find a rapid suppression of α1(II) collagen expression by RA, both at the protein and mRNA level, while α1(I) and α1(III) collagen synthesis and mRNA levels rise after 2–4 days of RA treatment. Loss of type II collagen synthesis and onset of type I collagen synthesis was also shown by immunofluorescence analysis (data not shown). The modulation of collagen gene expression was associated with a change in the chondrocyte phenotype from polygonal cells to stellate, fibroblast-like cells which no longer deposited extracellular cartilage matrix [see also Benya and Padilla, 1986; Yasui et al., 1986].

There is a difference, however, in the stimulation of α2(I) collagen synthesis by RA between chick sternal chondrocytes and rabbit articular chondrocytes: while Benya and Padilla [1986] reported the stimulation of $(\alpha1(I))_3$ collagen synthesis but not of α2(I) by RA using the rabbit chondrocytes, we found a dramatic enhancement of α2(I) mRNA and protein synthesis after 3–5 days of RA treatment in cultures of chick sternal chondrocytes. Similar findings were reported by Yasui et al. [1986] at the protein level and by Oettinger and Pacifici [1990] at the mRNA level. In accordance with a report of Saxe et al. [1985], we also find significant amounts of

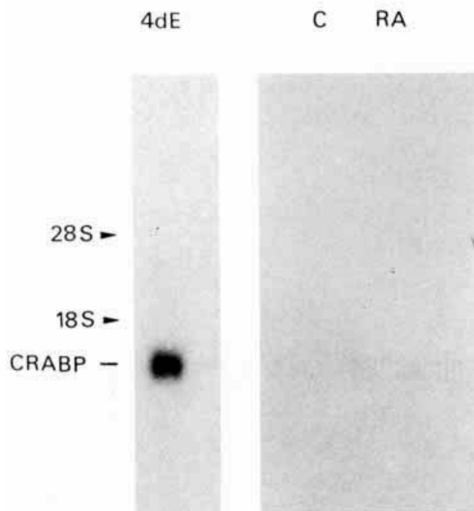


**Fig. 10.** Northern blot analysis of CRABP mRNA in 4-day-old chicken embryos, and freshly isolated (control) and dedifferentiated chondrocytes. Total RNA (20 μg) from 4-day-old chicken embryos (4dE) and 5 μg poly $A^+$ RNA from each control chondrocytes (C) and chondrocytes treated for 6 days with RA were blotted onto nylon membranes and hybridized to pCRABP296. Filters were exposed for 1 week.

untranslated α2(I) mRNA in normal chondrocytes.

Reports on the presence of untranslated α1(I) mRNA in chicken chondrocytes seen by Northern blot hybridization [Focht and Adams, 1984; Duchene et al., 1982], by RNase protection assays [Gerstenfeld et al., 1989], by in situ hybridization [Oshima et al., 1989], or by nuclear run-on experiments [Askew et al., 1991], and the rapid onset of translatable α1(I) mRNA synthesis by RA prompted us to investigate possible post-transcriptional control mechanisms of α1(I) expression in chondrocytes. In view of critical reports suggesting that the hybridization signals obtained with α1(I) cDNA probes in RNA from freshly isolated chicken chondrocytes might be due to cross-hybridization with α1(II) mRNA [Finer et al., 1985], and other studies showing no indication for the presence of α1(I) mRNA in chicken sternal chondrocytes although for untranslated α2(I), Saxe et al. [1985], we made attempts to obtain conclusive evidence on the presence or absence of untranslated α1(I) mRNA in chick sternal chondrocytes.

Initial experiments using the pCT200 probe coding for the 3′ end of pro α1(I) mRNA showed significant levels of α1(I) mRNA in freshly isolated chondrocytes, confirming studies by Duchene et al. [1982], Focht and Adams [1984], and Askew et al. [1991]. Sequence comparison of the pCT200 cDNA clone located in the pCO13 clone [Yamamoto et al., 1980] or pCG45 [Fuller and Boedtker, 1981] in the 3′ region of the COL1A1 gene with the corresponding region of the COL2A1 gene showed a stretch of 88 bp with only eight mismatches, (i.e., 90% sequence identity). Thus, previous studies reporting high levels of "untranslated" α1(I) mRNA in hyaline chondrocytes by Northern hybridization [Duchene et al., 1982; Focht and Adams, 1984] or by nuclear run on assays [Askew et al., 1991] may have to be reconsidered in view of this high chance of crosshybridization of 3′-derived probes to α1(II) mRNA. Biochemically, or by immunofluorescence, however, we could not detect any appreciable amount of α1(I) collagen synthesis in freshly isolated chondrocytes. In view of the high sequence homology between α1(I) and α1(II) mRNA in the 3′ and triple helical region, a cDNA fragment covering the first exon of α1(I) mRNA, which has the lowest homology to the corresponding exon of α1(II) mRNA, was amplified by PCR. This probe showed considerably lower yet significant hybridization with chondrocyte RNA. By RNase protection assays we verified this signal to be due to α1(I) mRNA.

To confirm this finding, in situ hybridization studies were performed in chick sternal cartilage, using $^{35}$S-radiolabeled antisense RNA transcribed from p1E1. Contrary to our expectation and in contrast to the finding by Oshima et al. [1989], there was no signal above background in the chondrocytes; only perichondrial cells contained significant amounts of α1(I) mRNA. Although the sensitivity of this method is lower than that of Northern blot hybridization and of RNase protection assays, this finding suggests that the low level of α1(I) mRNA found in freshly isolated chondrocytes may be due to perichondral cells remaining at the periphery of sternal cartilage, even after careful dissection and trypsin pretreatment.

In conclusion, our findings do not support the concept of post-transcriptional regulation of collagen expression in normal or RA treated chondrocytes. In accordance with previous findings by Yasui et al. [1986] and Horton et al. [1987], we confirm that RA at a concentration of 3 μM blocks transcription of the cartilage-specific collagens II, IX, and X and induces the transcription of type I and III collagen genes, as well as their translation into protein.

The effect of RA on the collagen gene expression in chondrocytes not only varies between

species, but also it depends on a number of other factors, including RA concentration and the age and origin of the chondrocytes. The genes of type II, IX, and X collagen respond to RA in a different manner. Recently, Oettinger and Pacifici [1990] reported a transient upregulation of type X collagen expression in chondrocyte cultures of 12-day-old chick vertebrae by 1 μM RA within 24–48 h of treatment; this was followed by a decline after 3–5 days. Furthermore, Sanchez et al. [1991] reported a suppression of collagen type II and IX mRNA by 0.5 mM RA, with reexpression of type II and X collagen within 5 and 15 days, respectively, after RA removal. In contrast, we observed a rapid decrease of type X collagen expression after 24 h treatment with 3 μM RA, with a moderate reappearence after 8 days of RA treatment (data not shown). The vertebral chondrocytes used by Oettinger and Pacifici [1990] had been selected as floaters and were grown for 12 days in culture during which time they became partially hypertrophic and synthesized type X collagen. Similarly, the sternal chondrocytes used in our system contained a significant number of hypertrophic chondrocytes derived from the cephalic portion of the xyphoid process which contain type X collagen mRNA. However, an important difference between the two studies is the concentration of RA used: 1 μM in the study by Oettinger and Pacifici, and 3 μM in our cultures. The importance of the dose dependence of the RA effect on chondrocytes and on chondrogenesis is well documented [Lewis et al., 1978]. It acts as a teratogen at high doses [Kochhar 1967, 1973], but also as a potent morphogen inducing the formation of limb cartilage at very low doses [Summerbell, 1983; Tickle et al., 1982]. Recent reports describe the action of very low dosage treatments (0.3–300 ng/ml) [Enomoto et al., 1990; Takishita et al., 1990], showing a primarily proliferative effect without affecting collagen I synthesis or induction of collagen I synthesis.

Several proteins are known to mediate the effects of RA. CRABP is expressed from day 3 to day 5 of chick development [Vaessen et al., 1990] and forms a gradient in the tip of the limb bud with its maximum in the anterior part. It presumably influences gene transcription by regulating the effective concentration of RA that reaches the nucleus [Maden et al., 1988]. Furthermore, the levels of CRABP II mRNA have been shown to increase in embryonic carcinoma (EC) cells in response to RA treatment [Wei et al., 1989; Giguere et al., 1990]. By Northern blot hybridization, we could not identify CRABP mRNA in chicken sternal chondrocytes or in RA induced dedifferentiated chondrocytes, indicating that this process is independent from CRABP expression and that the gene for the CRABP is not activated in chondrocytes after RA treatment at a concentration of 3 μM.

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