α2(I) collagen gene expression is up-regulated in quail chondrocytes pretreated with retinoic acid

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α2(I) collagen gene expression is induced in quail embryo chondrocytes pretreated with retinoic acid (RA). The initial appearance of α2(I) mRNA occurs around day 3 of culture in RA-free medium and rapidly progresses over the next 4 days. In transient transfection assays, expression of *COL1A2–CAT*, a chimeric gene bearing 3500 bp upstream the bone/tendon transcription start site from the human α2(I) gene fused to the *CAT* gene, is stimulated severalfold in RA-treated chondrocytes. In contrast, enzyme activity is very low in untreated chondrocytes, suggesting that the sequences required for RA-induced transcription of the α2(I) gene are present in this plasmid. Analysis of α2(I) promoter sequences performed with deletion mutants gives overlapping results in collagen type I-producing fibroblasts and chondrocytes withdrawn from RA treatment. These experiments suggest that RA-induced transcription of the α2(I) collagen gene in chondrocytes is regulated by the binding of transcription factors to the same regulatory sequences that control transcription in fibroblasts.

INTRODUCTION

Type I collagen, a heterotrimer composed of two α1 chains and one α2 chain, is present in most tissues of mesenchymal origin, such as skin, tendon and bone. In bone, 90% of the organic matrix is collagen type I and in cartilage 40% is collagen type II. Expression of collagen types I and II is tightly regulated during chondrogenesis (Mayne and Burgeson, 1987; Ramirez and Di Liberto, 1990; Vuorio and de Crombrugghe, 1990). As prechondrogenic cells differentiate into chondrocytes, they stop synthesizing collagen type I and initiate synthesis of collagen type II (von der Mark, 1980). Despite the presence of appreciable amounts of type I mRNA, there is little or no detectable synthesis of type I chains in either cartilage or mature chondrocytes grown in suspension (Pawlowski et al., 1981; Focht and Adams, 1984; Finer et al., 1985; Saxe et al., 1985). Chondrocyte α1(I) collagen mRNA remains in the nucleus in an unprocessed form and is not available for protein synthesis (Saxe et al., 1985; Allebach et al., 1985). The cessation of α2(I) collagen synthesis results from a switch in promoter utilization from the bone/tendon promoter to the cartilage promoter (Bennett and Adams, 1990). The cartilage promoter lies in the second intron of the gene and directs the transcription of a shorter mRNA which no longer encodes α2(I) collagen, thus explaining the absence of α2(I) collagen from cartilage and chondrocytes grown in suspension (Bennett et al., 1989; Bennett and Adams, 1990).

α2(I) gene expression can be induced in several experimental systems. In fact, transformation with Rous sarcoma virus (RSV) (Adams et al., 1982; Gionti et al., 1983, 1989; Allebach et al., 1985) or treatment of chondrocytes grown in suspension with the tumour promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Finer et al., 1985), inhibits the chondrocyte phenotype so that collagen type II and other cartilage-specific products are no longer synthesized and induces expression of α2(I) collagen along with fibronectin and cell adhesion. Bromodeoxyuridine (BrdUrd) treatment also inhibits the chondrocyte phenotype and induces expression of collagen type I along with fibronectin and cell adhesion (Saxe et al., 1985; Askew et al., 1991). In this case, the induction of the α2(I) collagen gene is the result of utilization of the bone/tendon promoter in that the 5′ end of α2(I) RNA in untreated chondrocytes differs from that present in BrdUrd-shifted chondrocytes (Askew et al., 1991). Moreover, Beck et al. (1991) identified in chondrocyte chromatin a cartilage hypersensitive site in the second intron of the α2(I) gene. This site is lost with BrdUrd treatment, whereas the fibroblast hypersensitive site, previously shown to be associated with the active transcription of this gene in fibroblasts, is not present in chondrocyte chromatin and is present in BrdUrd-shifted chondrocytes (Beck et al., 1991).

The induction of the α2(I) gene along with suppression of the chondrocyte phenotype has been shown to occur in chick vertebral chondrocytes on treatment with retinoic acid (RA) (Yasui et al., 1986; Oettinger and Pacifici, 1990).

RA is a signalling molecule apparently involved in a variety of morphogenetic processes such as patterning of developing and regenerating vertebrate limbs [for reviews see Eichele (1989) and De Luca (1991)]. The cellular responses to retinoids are primarily mediated by a complex system of cytoplasmic binding proteins and nuclear receptors (De Luca, 1991; Gudas, 1992). RA is a well-known inhibitor of the chondrocyte phenotype in cultured cartilage cells (Benya and Padilla, 1986; Horton and Hassel, 1986; Yasui et al., 1986; Horton et al., 1987; Benya et al., 1988).

Here we present data dealing with the induction of the α2(I) collagen gene in chondrocytes pretreated with RA and allowed to recover the expression of collagen type II by culturing them in the absence of RA for an additional week. Primary cultures of embryonic quail chondrocytes synthesize the specialized products characteristic of differentiated chondrocytes, including collagen type II (Gionti et al., 1985). Exposure of these cells to low levels of RA for 3 days causes them to lose their differentiated

Abbreviations used: RA, retinoic acid; TPA, 12-*O*-tetradecanoyl:phorbol 13-acetate; BrdUrd, 5-bromo-2′-deoxyuridine; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; COL1A2, α2 chain of collagen type I; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 1 × SSPE, 1.2 mM NaCl/10 mM sodium phosphate buffer (pH 7.4)/1 mM Na_2EDTA.

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phenotype. The previously floating chondrocytes attach to the bottom of the culture dish, synthesize fibronectin and assume a fibroblastic shape. We have shown that RA-treated chondrocytes can re-express collagen type II if they are cultured in the absence of RA for an additional week (Sanchez et al., 1991). Once withdrawn from RA-containing medium, cells continue to synthesize fibronectin, keep growing as attached cells and assume an epithelial-like morphology characteristic of chondrocytes grown in monolayer. Therefore fibronectin and cell adhesion are irreversibly induced by RA treatment. In this study, we investigated the induction of α2(I) expression in chondrocytes pretreated with RA. We tested the transcriptional activity of the *COL1A2–CAT* plasmid in which 3500 bp upstream the bone/tendon transcription start site from human α2(I) collagen gene drives the expression of the *CAT* gene without any contribution from intronic sequences. This region is active as a promoter in transfection assays on chondrocytes withdrawn from RA treatment. Chloramphenicol acetyltransferase (CAT) activity is nearly undetectable in control untreated chondrocytes transfected with this chimeric gene. These results suggest that the sequences required for RA-induced transcription of the α2(I) gene in chondrocytes are present in this plasmid.

To assess the contribution of *cis*-acting sequences to RA-induced transcription, we analysed by transient transfection assay the expression of chimeric genes obtained by introducing progressive deletions at the 5′ end of *COL1A2–CAT* in both fibroblasts and chondrocytes withdrawn from RA treatment. We observed that *cis*-acting elements in the promoter sequences displayed either a positive or negative influence regardless of the cell type, suggesting that RA-induced transcription of *COL1A2* gene in chondrocytes is controlled by binding of *trans*-acting factors to the same regulatory elements already identified in fibroblasts by Boast et al. (1990).

EXPERIMENTAL

Cell culture

Chondrocytes were isolated from day- 10 quail embryo tibiae as previously described (Gionti et al., 1985). Floating cells were resuspended in Coon's modified F12 medium (Ambesi-Impiombabo et al., 1980) supplemented with 10% fetal calf serum; after addition of RA, cells were plated at 1×10^5 cells/ml. All-*trans*-RA (Eastmann-Kodak) was dissolved in 95% ethanol and stored at −80 °C in the dark. This solution was diluted with growth medium on day 0 and control cultures received an equivalent amount of ethanol. Chondrocytes were treated with RA for 72 h and subsequently cultured in normal medium for about 7 days. The experiments reported were performed on chondrocytes exposed to RA at 0.5 μM; no toxic effect was ever detected at this concentration in treated cultures.

DNA probes

The α1(II) probe used was pYN 2142, harbouring a 1200 bp cDNA insert coding for the C-propeptide of chicken type II collagen (Ninomiya et al., 1984). pYN 535 was a 760 bp cDNA fragment coding for the C-propeptide of chicken α2(I) collagen (Ninomiya and Olsen, 1984). The GAPDH probe was a full-length cDNA clone encoding rat glyceraldehyde 3-phosphate dehydrogenase (Fort et al., 1985).

RNA extraction and Northern-blot hybridization

Total cellular RNA, extracted by the method of Chomczynski and Sacchi (1987), was denatured, fractionated on formaldehyde–agarose gels (Lehrach et al., 1977) and blotted on to an Amersham Hybond N nylon membrane.

Hybridization was performed according to the manufacturer's (Amersham) directions, with the following modifications: the hybridization temperature was 42 °C; filters were washed twice in 2 × SSPE/0.1% SDS for 5 min at room temperature and then five times in 0.2 × SSPE/0.1% SDS at 55 °C. Washed filters were dried and exposed to Fuji films with intensifying screens at −80 °C. Autoradiographs were scanned with a 2202 Ultrascan laser densitometer (LKB).

High-specific-activity random-primed probes were prepared with Amersham kits as specified by the supplier.

Chimeric DNA constructs

COL1A2–CAT (formerly pMS-3.5/CAT) contains *COL1A2* proximal promoter fused to the *CAT* gene and was derived from a 3.5 kb *Eco*RI–*Sph*I genomic subclone which spans from position −3500 to +58 of the *COL1A2* 5′-flanking sequences (Dickson et al., 1985). Deletion mutants of *COL1A2–CAT* were generated using different restriction sites conveniently located upstream of the canonical CCAAT (−83 to −79) and TATA (−33 to −28) boxes (see Figure 4) (Boast et al., 1990).

Transfection experiments

Cells were transfected by either the calcium phosphate technique followed by a 15% glycerol shock after 4 h or the liposome technique according to the manufacturer's directions (Boehringer, DOTAP transfection reagent). In the first case, cells were treated with trypsin 24 h before transfection and seeded at a density of 1×10^6 cells/60 mm dish; in the second case transfection was performed on a 1×10^6 suspension of cells (when dealing with chondrocytes grown in suspension, the liposome technique was used). Calcium phosphate precipitates were prepared as described by Graham and Van der Eb (1973) with plasmid DNA at a concentration of 5 μg/ml. The total amount of test DNA was 5 μg regardless of the technique used. Parallel cultures were transfected with 1 or 5 μg respectively of *RSV–CAT* as reference plasmid (Gorman et al., 1982a) depending on whether the calcium phosphate or liposome technique was used. Less DNA gives no appreciable transfection efficiency with the DOTAP reagent. When appropriate, fresh medium with RA was added after transfection. Cells were analysed for CAT activity after 48–72 h and cell extracts were assayed for conversion of ^{14}C-labelled chloramphenicol into acetylated chloramphenicol by t.l.c. coupled with fluorography by the method of Gorman et al. (1982b). Multiple DNA preparations were used for each experiment and care was taken to ensure that all assays were carried out in the linear phase. In control and RA-treated chondrocytes, *COL1A2–CAT* expression displays a linear relationship with increasing amounts of protein (not shown). Parallel transfections with the reference plasmid were performed in all experiments.

RESULTS

In this study we investigated the induction of α2(I) expression in quail chondrocytes pretreated with RA. RA treatment rapidly inhibits expression of the chondrocyte phenotype while inducing fibronectin expression, cell adhesion and a fibroblast-like morphology in floating chondrocytes. In a previous study, we have shown that RA-treated chondrocytes can completely recover the ability to synthesize collagen type II if they are allowed to grow in the absence of RA for about 2 weeks (Sanchez et al., 1991).

Total cellular RNA extracted from untreated floating

Table 1 Relative levels of specific collagen mRNAs in quail embryo chondrocytes and fibroblasts

Collagen mRNA levels calculated from densitometric scannings were normalized to GAPDH expression. α1(II) expression in control chondrocytes was set at 1; the same setting was used for α2(I) expression in fibroblasts. —, not detectable.

	Relative levels of collagen mRNA			
		α2(I)		
Cell type	α1(II) Expt. 1	Expt. 1	Expt. 2	Expt. 3
Control untreated chondrocytes	1.00	–	–	–
Chondrocytes treated with RA for 3 days	0.03	–	–	–
Treated chondrocytes 3 days after removal of RA	0.01	0.0015	0.018	0.004
Treated chondrocytes 6 days after removal of RA	0.40	0.05	0.06	0.05
Control fibroblasts	–	1.00	1.00	1.00

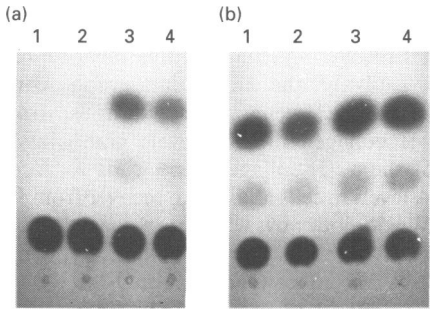

Figure 1 Tissue specificity of *COL1A2* proximal promoter

Representative CAT assays of *COL1A2–CAT* plasmid transfected into (**a**) control untreated chondrocytes and (**b**) quail embryo fibroblasts. In each autoradiogram are, in duplicate, from left to right, CAT assays of *COL1A2–CAT* (lanes 1 and 2) and *RSV–CAT* (lanes 3 and 4) plasmids. Collagen-driven CAT activity was expressed as the relative percentage with respect to CAT conversions of the reference plasmid *RSV–CAT*. The ratio between the collagen-driven CAT activities in fibroblasts and chondrocytes is 24-fold. Transfections were performed by the calcium phosphate technique.

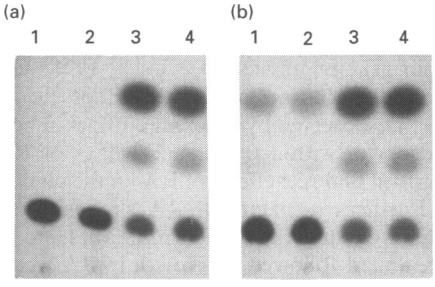

Figure 2 RA-induced transcription activity of *COL1A2–CAT* in chondrocytes

Representative CAT assays of the *COL1A2–CAT* plasmid transfected into control untreated chondrocytes (**a**) and chondrocytes recovering from RA treatment for 6 days (**b**). In each autoradiogram are, in duplicate, from left to right, CAT assays of *COL1A2–CAT* (lanes 1 and 2) and *RSV–CAT* (lanes 3 and 4) plasmids. Collagen-driven CAT activity was expressed as the relative percentage with respect to CAT conversions of the reference plasmid *RSV–CAT*. The ratio between the collagen-driven CAT activities in recovering chondrocytes and control chondrocyte is 7-fold. Transfections were performed by the liposome technique.

chondrocytes, from chondrocytes undergoing RA treatment, from chondrocytes pretreated with RA but grown in its absence for 3 and 7 days, and from quail embryo fibroblasts (collagen-type-I-producing cells) was analysed by Northern-blot hybridization. Collagen mRNA levels calculated from densitometric scanning and normalized to GAPDH expression are shown in Table 1. This analysis shows the accumulation of α2(I) collagen mRNA in chondrocytes recovering from RA treatment. The initial appearance of α2(I) mRNA occurs around day 3 of culture without RA and rapidly progresses over the next 4 days. On RA treatment, α1(II) mRNA levels decrease sharply; on day 7 of culture without RA, cells re-express this collagen, although in lower amounts than untreated floating chondrocytes. The accumulation of α2(I) mRNA is accompanied by a 3-fold stimulation of alkaline phosphatase activity (not shown).

In order to find out which kind of promoter is involved in RA-induced up-regulation of the α2(I) gene, we performed transient transfection experiments with *COL1A2–CAT*, a construct in which 3500 bp upstream of the fibroblast transcription start site of α2(I) human gene drives *CAT* gene expression without any contribution from intronic sequences. Ramirez and co-workers (Boast et al., 1990) have shown that this region contains all the sequences necessary for cell-specific expression in that it is able to direct the production of high levels of CAT protein in collagen-type-I-producing fibroblasts of either human or avian origin. CAT activity is nearly undetectable in cells not producing collagen type I. *COL1A2–CAT* was transiently expressed in quail fibroblasts and control untreated chondrocytes. Parallel cultures were transfected with *RSV–CAT* and the collagen-driven CAT activity was expressed as the relative percentage with respect to CAT conversion obtained with the *RSV–CAT* reference plasmid. As expected [see also Boast et al. (1990)], *COL1A2–CAT* is readily expressed in fibroblasts and weakly expressed in chondrocytes (Figure 1), the difference in the relative ratios of CAT activity in this experiment being 24-fold.

We next analysed *COL1A2–CAT* expression in control and RA-treated chondrocytes (Figure 2). Several independent transfection experiments were performed. In this particular experiment, the chimeric gene expresses 7-fold higher levels of CAT activity in chondrocytes allowed to recover from RA treatment for 6 days than in untreated floating chondrocytes; in other experiments, the RA-induced stimulation of CAT activity observed was 10-fold. CAT activity is also low in chondrocytes undergoing RA treatment (not shown).

We also performed some transient transfection experiments on chick tibial chondrocytes growing in monolayer. In four independent experiments we detected an RA-induced stimulation of CAT activity ranging from 3.1- to 3.8-fold in chick chondrocytes allowed to recover from RA treatment for 7 days as compared with control untreated chondrocytes (not shown). Here again, no stimulation of CAT activity was detected in chondrocytes undergoing RA treatment. The RA-induced stimulation of CAT activity that we detected in chick chondrocytes is smaller than that detected in quail chondrocytes perhaps because of the higher level of expression of the endogenous gene in untreated chick chondrocytes, as these cells grow in monolayer and more easily dedifferentiate in culture. In line with these results, Bennett et al. (1989) have shown that α2(I) mRNA from chick chondrocytes grown in monolayer display both the cartilage and the bone/tendon-specific 5′ end, the transcription also being driven by the fibroblast promoter (Bennett and Adams, 1990). Thus RA treatment induces α2(I) gene transcription regardless of the cell shape of the starting chondrocyte population, and the sequences required for RA-induced transcription of this gene are present in the *COL1A2* proximal promoter.

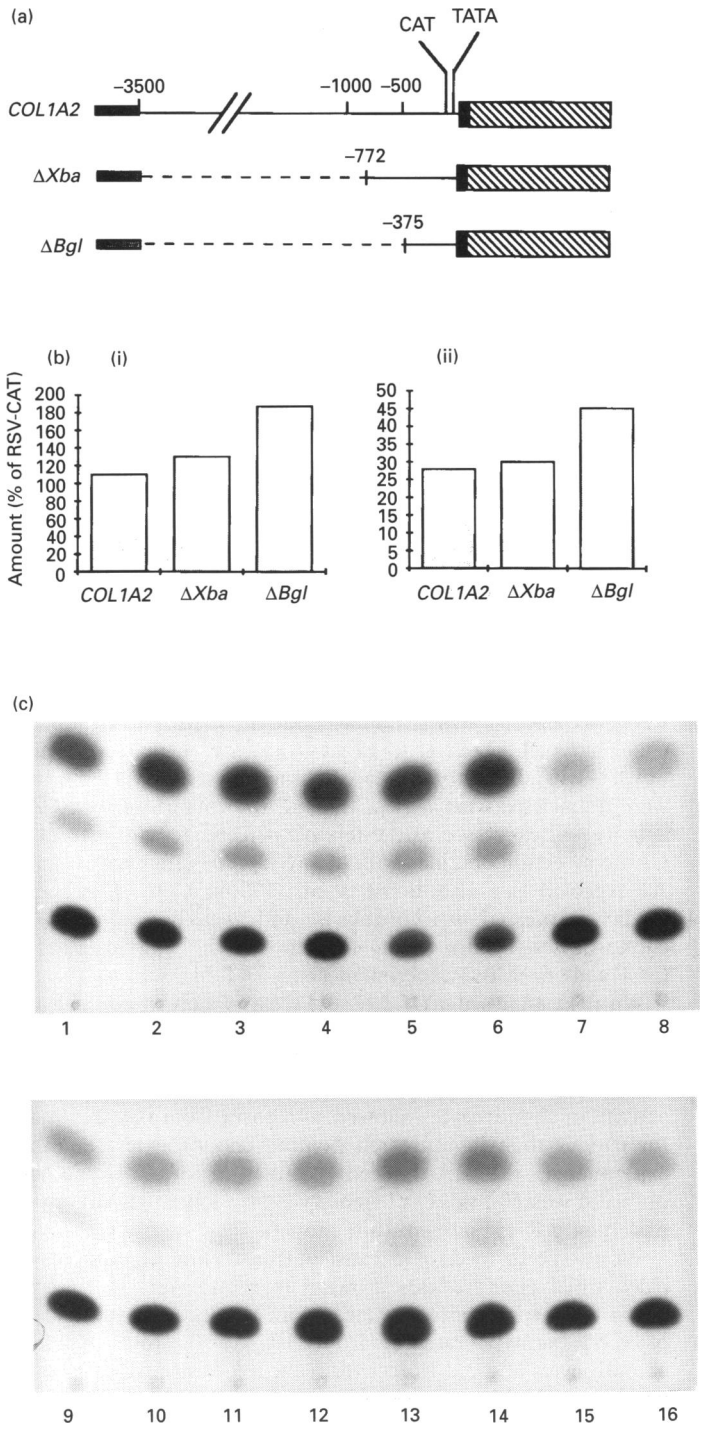

Figure 3 Deletion analysis of *COL1A2* promoter

(**a**) Schematic representation of 5′-flanking sequences of *COL1A2* gene with the relative position of *Xba*I and *Bgl*II restriction sites utilized for the generation of the relevant deletion mutants, Δ*Xba* and Δ*Bgl*. The black box signifies the first 58 bp of exon 1 of *COL1A2*, whereas the hatched box represents the *CAT*. (**b**) Histograms indicating the percentage CAT conversion of the three chimeric constructs relative to *RSV–CAT*. All transfections were performed by the calcium phosphate technique on fibroblasts (i) and chondrocytes withdrawn from RA-containing medium for 6 days (ii). (**c**) Representative CAT assays of *COL1A2–CAT* and its deletion mutants. Quail embryo fibroblasts (1–8) and RA-treated chondrocytes (lanes 9–16) were transfected with the three chimeric constructs as indicated in (**b**). Lanes 1, 2, 9 and 10, cells transfected with the *COL1A2–CAT* plasmid; lanes 3, 4, 11 and 12, cells transfected with Δ*Xba* deletion mutant; lanes 5, 6, 13 and 14, cells transfected with Δ*Bgl* deletion mutant; lanes 7, 8, 15, 16, cells transfected with *RSV–CAT*. All assays were incubated for 60 min at 37 °C except those relevant to *RSV–CAT*-transfected cells which were incubated for 15 min.

To study the contribution of promoter sequences to α2(I) transcription induced by RA treatment, we transfected fibroblasts and recovering quail chondrocytes with some *COL1A2–CAT* deletion mutants. The mutants were obtained by introducing progressive deletions at the 5′ end of the gene leaving only either 772 bp or 376 bp of the promoter sequence fused to the *CAT* gene (Boast et al., 1990). Expression of such mutants in untreated control cells did not vary from the background level and is not shown. Figure 3 shows collagen-driven CAT activity in fibroblasts and RA-treated chondrocytes transfected with the wild-type fusion gene and its deletion mutants. The behaviour of such mutants in these two cell types is quite comparable. Deletion of the fragment Δ*Xba–*Δ*Bgl*, which removes the sequences spanning from −772 to −376, may eliminate putative negative elements [see also Boast et al. (1990)] in that CAT activity is somehow increased in both cell types. A third mutant in which all the sequences before nucleotide −108 are deleted, leaving the canonical CCAAT (−83 to −79) and TATA box (−33 to −28) was tested. In this case, collagen-driven CAT activity fell to the background level in both fibroblasts and treated chondrocytes (results not shown) [see also Boast et al. (1990)].

DISCUSSION

Chondrocytes recovering from RA treatment represent a useful system for studying the molecular events controlling the expression of the α2(I) gene. After withdrawal from RA treatment, type II collagen mRNA levels gradually increase and activation of the α2(I) collagen gene leads to accumulation of α2(I) mRNA levels in cells allowed to recover from RA treatment for 7 days. We do not know whether in our culture the same cell is able to produce both types of collagen or whether we are dealing with a mixed cell population. By 7 days after RA removal, chondrocytes are polygonal and heterogeneous in size, as some scattered hypertrophic cells are present. RA treatment also leads to increased alkaline phosphatase activity, maximal stimulation occurring 5–7 days after RA removal.

A similar shift in collagen types has been reported in chick chondrocytes growing in monolayer undergoing long-lasting RA treatment (Yasui et al., 1986; Oettinger and Pacifici, 1990; Pacifici et al., 1991). Pacifici and co-workers observed a 5-fold increase in steady-state levels of α2(I) mRNA on day 5 of culture in 1 μM RA. Long-lasting treatments with other agents, e.g. BrdUrd and TPA, are also required before induction of this gene can be observed (Finer et al., 1985; Saxe et al., 1985; Yasui et al., 1986; Askew et al., 1991; Beck et al., 1991). RA is known to rapidly reach the nucleus, activate its nuclear receptors and lead to changes in gene expression (De Luca, 1991; Gudas, 1992). Thus the rapid inhibition of expression of the chondrocyte phenotype so far observed by different authors in both chick and quail chondrocytes within the first 2–3 days of RA treatment could be ascribed to direct effects of RA on the regulation of gene expression. Further treatment with RA is required to detect α2(I) gene induction (Oettinger and Pacifici, 1990). Here we show that a shorter treatment followed by growth in RA-free medium for an additional 7 days leads equally well to α2(I) gene induction in quail chondrocytes. The timing of each change could reflect differences in sensitivity of each gene to RA or the above-mentioned agents. Alternatively, induction of the α2(I) gene could be a secondary effect of the inductive agent, i.e. the timing of each change could reflect a distinction between primary and secondary effects, the first ones being the modulated expression of fibronectin and cartilage matrix genes and the alteration of cell-surface receptors for matrix protein. These primary changes

could in turn influence cell adhesion and differentiation pathways thus possibly leading to stimulation of α2(I) gene expression.

In order to find out whether the fibroblast promoter is involved in the transcriptional activation of the α2(I) gene, we performed transient transfection assays in control and RA-treated chondrocytes with *COL1A2–CAT*, a chimeric gene in which the usual fibroblast promoter from the human α2(I) gene drives the expression of the *CAT* gene without any contribution from intronic sequences. In transient expression assays, CAT activity is very low in control chondrocytes and chondrocytes undergoing RA treatment. *COL1A2–CAT* causes the expression of high levels of CAT activity in recovering chondrocytes, indicating that the regulatory elements required for activation of RA-induced transcription are present in this plasmid. These results suggest that a change in the transcription start site could be the regulatory mechanism involved in the induction of *COL1A2* gene expression in RA-treated chondrocytes. *COL1A2–CAT* has been shown to contain all the sequences necessary to direct abundant cell-specific transcription in type-I-collagen-producing cells such as fibroblasts (Boast et al., 1990). Deletion experiments have narrowed down the active segment of the human promoter to a phylogenetically conserved sequence between nucleosides −376 and −108 relative to the transcription start site (Boast et al., 1990). To test the functional consequence of progressive deletions of *cis*-acting sequences from the 5′ end, transient transfection assays with some deletion mutants were performed in fibroblasts and recovering chondrocytes. *Cis*-acting elements in the 5′ upstream sequences display either a positive or negative influence regardless of the cell type, indicating that transcription of this collagen gene is controlled by the binding of *trans*-acting factors to the same regulatory motifs in both fibroblasts and chondrocytes recovering from RA treatment.

The same regulatory mechanisms, operating in the reverse direction to turn off the expression of the α2(I) gene as precursor mesenchyme cells differentiate into chondrocytes, appear to work during the induction of this gene in mature chondrocytes treated with RA. The physiological role of the altered chondrocyte transcript as well as the mechanism that effects the change in the transcription start site are at present unknown.

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