Multiple Negative Elements in a Gene That Codes for an Extracellular Matrix Protein, Collagen X, Restrict Expression to Hypertrophic Chondrocytes

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Abstract. During skeletal development, chondrocytes go through several stages of differentiation. The last stage, chondrocyte hypertrophy, occurs in areas of endochondral ossification. Mature hypertrophic chondrocytes differ from immature chondrocytes in that they become postmitotic, increase their cellular volume up to eightfold, and synthesize a unique set of matrix molecules. One such molecule is a short collagenous protein, collagen X. Previous studies have shown that collagen X is not expressed by other cell types and that its specific expression in hypertrophic chondrocytes is controlled by transcriptional mechanisms. To define these mechanisms, plasmid constructs containing the chicken collagen X gene promoter and 5′ flanking regions fused to a reporter gene (chloramphenicol acetyl transferase, CAT) were transfected into primary cultures of collagen X–expressing and nonexpressing cells. A construct containing a short (558 bp) promoter exhibited high levels of CAT activity in all cell types (fibroblasts, immature, and hypertrophic chondrocytes). Adding a 4.2-kb fragment of 5′ flanking DNA to this construct resulted in a dramatic reduction of CAT activity in fibroblasts and immature chondrocytes, but had no effect in hypertrophic chondrocytes. Addition of three subfragments of the 4.2-kb fragment to the initial construct, either individually or in various combinations, showed that all subfragments reduced CAT activity somewhat in non-collagen X-expressing cells, and that their effects were additive. Unrelated DNA had no effect on CAT activity. The results suggest that multiple, diffuse upstream negative regulatory elements act in an additive manner to restrict transcription of the collagen X gene to hypertrophic chondrocytes.

THE transition from cartilage to bone during skeletal development is a multi-step process involving cartilage synthesis, hypertrophy, calcification, degradation, and replacement of cartilage by bone and marrow. The primary growth plate of developing long bones, located between the epiphyseal cartilage and the bone of the metaphysis, offers an excellent display of this sequence of events as it relates to cell differentiation (Poole, 1991). The reserve, or resting zone, just adjacent to the epiphyseal cartilage, consists of nondividing, small, spherical cells. These cells give rise to proliferating chondrocytes which appear as flattened cells stacked in columns within the proliferating zone. These chondrocytes become round and enlarge into hypertrophic chondrocytes within the zone of maturation. The cells finally enter the upper and lower hypertrophic zones, the latter being distinguished from the former by the presence of calcifying zones of cartilage. The entire differentiation–maturation process of a single cell takes ~3 d (Poole, 1991). Permanent cartilage, which does not undergo endochondral ossification, contains small, round chondrocytes which produce the components of cartilage matrix, particularly type II collagen and aggrecan. In contrast, hypertrophic chondrocytes selectively synthesize collagen X, and thus cartilage which is undergoing endochondral ossification can be distinguished by the presence of hypertrophic chondrocytes and their unique collagen X product (Gibson et al., 1984; Schmid and Linsenmayer 1985*a*,*b*).

Collagen X, secreted by hypertrophic chondrocytes, is localized pericellularly in a capsule-like structure (Gibson et al., 1986; Haynes, 1990; Schmid and Linsenmayer, 1990; Lu Valle et al., 1992). It is also found in the extracellular matrix in the vicinity of collagen II–containing fibrils (Poole and Pidoux, 1989; Schmid and Linsenmayer, 1990). The hypertrophic chondrocyte-specific transcriptional activation of the collagen X gene (Castagnola et al., 1988; Lu Valle et al., 1989, 1992) is accompanied by decreases in the levels of both collagen IX mRNA (Linsenmayer et al., 1991) and collagen II mRNA (Oshima et al., 1989; Lu Valle et al., 1992). In fact, type collagen X constitutes ~45% of the total collagen produced by mature hypertrophic chondrocytes (Reginato et al., 1986), and therefore is a major secreted protein product of this cell type.

The chick embryonic sternum is an excellent tissue model in which to study the cell-specific control of collagen X gene

expression. The cephalic portion of the sternum begins to undergo endochondral ossification at embryonic day 16, when chondrocyte hypertrophy and collagen X gene expression are first observed (Gibson and Flint, 1985; Lu Valle et al., 1992). The caudal portion does not undergo endochondral ossification during embryonic and early postnatal development (Fell, 1956), but will ossify in the adult chicken. Chondrocytes isolated from the cephalic portion of sterna after embryonic day 16 are large in size and actively synthesize collagen X in culture. In contrast, chondrocytes from the caudal region at the same embryonic stage are initially small in size and do not synthesize collagen X; however, over a period of 2–5 wk in culture, these cells undergo maturation and develop into hypertrophic, collagen X–producing cells (Solursh et al., 1986; Castagnola et al., 1987; Pacifici et al., 1991*a*).

We wished to investigate the mechanisms by which the cell-specific transcriptional activation of the collagen X gene in hypertrophic chondrocytes is achieved. To determine which portions of the gene are involved in hypertrophic chondrocyte-specific promoter activity, we used a 5,300-bp DNA fragment which includes the first intron and extends 5′ in the chicken collagen X genomic clone (Lu Valle et al., 1988) to prepare collagen X promoter–reporter gene constructs. The reporter gene used in these constructs was the bacterial chloramphenicol acetyl transferase gene (CAT)[1] (Gorman et al., 1982). By introducing collagen X promoter-CAT constructs into primary cultures of chick embryonic collagen X-expressing hypertrophic chondrocytes (HC) and non-expressing immature chondrocytes (IC) and fibroblasts (CEF), we found that a 640-bp fragment containing the type X collagen transcription start site directed high levels of CAT activity in all cell types. Hypertrophic chondrocyte-specific transcriptional activity was generated by the presence of multiple negative regulatory elements within 4,200 bp upstream of the 640-bp fragment. These negative elements, when present together, reduced promoter activity by ~90% in non-type X collagen expressing cells, but had no significant effect on activity in hypertrophic chondrocytes.

Materials and Methods

Cell Culture, Immunocytochemistry, and Transfection

HC and IC were isolated from the upper one third and the lower one third of chick embryonic day 18 sterna, respectively, by digestion of minced tissue in 1.5 mg/ml collagenase type I (Sigma Immunochemicals, St. Louis, MO) and 0.1% trypsin (GIBCO BRL, Gaithersburg, MD) in DME for 1 h at 37°C. This first digestion removes perichondrial fibroblasts. This was followed by removal of the medium, addition of more medium containing the same concentrations of enzymes, and incubation for an additional 2 h. Cells were filtered through Nytex, rinsed in DME containing 10% FCS (Hyclone Labs, Logan, UT), and plated at 3×10^5 cells/ml of high glucose DME containing 10% FCS and 50 U/ml penicillin and streptomycin (complete medium). Chondrocytes were maintained in primary culture for 3–7 d before transfection. CEF were obtained by digestion of tendon or skin from day 12–19 embryos with trypsin/collagenase as above in DME for 30 min. Resultant cells were plated at 1×10^5 cells/ml in the same medium as above.

Cells were processed for immunofluorescence as described (Pacifici et al., 1983). The antiserum used in these assays has been demonstrated to be specifically against chicken collagen X (Pacifici et al., 1991*b*).

Transient transfections were performed using either a high-efficiency calcium phosphate coprecipitation method in the presence of N,N-*bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (Chen and Okayama, 1987) or liposome-mediated transfection using DOTAP (Boehringer Mannheim Corp., Indianapolis, IN). In both cases, chondrocytes were treated with 4 U/ml bovine testes hyaluronidase (Calbiochem-Novabiochem Corp., La Jolla, CA) during the 24 h before transfection as well as during the incubation with DNA. We have found that this treatment, which removes pericellular proteoglycans (Pacifici et al., 1983), increases transfection efficiency four- to sixfold on the basis of either CAT activity or number of β-galactosidase positive cells. Each 60-mm dish of cells was cotransfected with 4 μg of the collagen X promoter-CAT vector constructs and 1 μg of the β-galactosidase plasmid pCH110 (Hall et al., 1983) for a period of 8–14 h. The medium was then replaced and cells were incubated for an additional 48 h before harvest.

Collagen X Promoter-CAT Plasmids

The CAT vectors used for these studies were pBLCAT2, which contains the herpes simplex virus (HSV) thymidine kinase promoter 5′ of the coding region for CAT and a polylinker 5′ of the promoter, and pBLCAT3, a promoterless vector which contains a polylinker 5′ of the CAT coding region (Luckow and Schutz, 1987). These plasmids were used as positive and negative controls for CAT activity, respectively. Collagen X promoter fragments (see Fig. 1) were cloned into the polylinker region of the CAT vectors, using restriction endonucleases (Boehringer Mannheim Corp.) and phosphorylated linkers (New England Biolabs Inc., Beverly, MA). The 640-bp fragment, which contains the transcription start site and 558 bp of 5′ flanking sequence in addition to 82 bp of the first (untranslated) exon, was excised from the chick genomic clone PL10 (Lu Valle et al., 1988) using restriction endonucleases HindIII and SacI. The addition of SalI linkers to the 3′ SacI site allowed the 640-bp fragment to be ligated in the correct orientation into the HindIII and SalI sites of the polylinker region of pBLCAT3. The C-640 fragment was excised from PL10 using NsiI and SacI. After the addition of SalI linkers, the fragment was ligated into the SalI site of the polylinker in pBLCAT3 and oriented using the internal HindIII site. The B fragment was excised from PL10 with PstI and NsiI, and was ligated into the PstI site of the polylinker of C-640 CAT. This PstI site is located just 5′ and adjacent to the SalI site in which the C-640-bp fragment was ligated. Since the 3′ NsiI site of the B fragment is compatible for ligation with PstI but does not regenerate a PstI site, digesting the resultant construct with PstI and HindIII (the internal site in the C-640 fragment) allowed the orientation of the B fragment to be determined. The A fragment, the AB fragment, and the ABC fragment, were all excised from PL10, modified with HindIII linkers, and ligated into the HindIII site adjacent and 5′ of the 640-bp fragment in pBLCAT3. Orientation was determined using internal restriction sites, except for the A fragment, which was oriented by sequence analysis. Unrelated DNA (3,300 bp of coding sequence from a nuclear pore complex protein, NP, a gift from Dr. B. Burke, Department of Cellular and Molecular Biology, Harvard Medical School) was ligated into the HindIII site 5′ of the 640-bp fragment in pBLCAT3 as an additional control construct. All plasmids were purified by polyethylene glycol precipitation (Lis, 1980) followed by cesium chloride/ethidium bromide equilibrium centrifugation (Radloff et al., 1967).

Sequencing was carried out as described (Lu Valle et al., 1988) using the dideoxy termination method (Sanger et al., 1977).

Type X Promoter Activity Assays

Cells were harvested after transfection according to Gorman et al. (1982) and were resuspended in 110 μl of 0.25 M Tris pH 7.8. After preparation of the cell lysates by three freeze/thaw cycles from ethanol/dry ice to 37°C, for 5 min each, and centrifugation (5 min at 4°C at 16,000 *g*), 40 μl of the extract was used in a β-galactosidase activity assay (Herbomel et al., 1984). Aliquots of cell lysate containing 5 U of β-galactosidase activity were used for CAT assays. CAT activity was determined by chromatographic assay according to Gorman et al. (1982), and quantitated by scintillation counting. Activity was calculated as:

$$\frac{\text{cpm acetylated [}^{14}\text{C]chloramphenicol}}{\text{cpm acetylated + cpm unacetylated [}^{14}\text{C]chloramphenicol}} \quad (1)$$

Values were expressed as percent of CAT activity in cells transfected with 640 CAT, where 640 CAT activity is 100%. Figures which display CAT ac-

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyl transferase; CEF, chick embryonic fibroblasts; HC, hypertrophic chondrocytes; HSV, herpes simplex virus; IC, immature chondrocytes.

tivity graphically are representative of several repetitions of the same experiment done in duplicate. Each value represents the average of duplicate transfections. The range of these values is stated in the figure legends.

Results

Effects of Proximal Flanking Sequences on the Ability of the Collagen X Promoter to Drive CAT Expression in HC and CEF

Initial transfection experiments compared the promoter activity of both 640 CAT (Fig. 1) and the positive control vector pBLCAT2 (Luckow and Schutz, 1987) in both collagen X-expressing HC (Fig. 2, *a* and *b*) and non-collagen X-expressing CEF (Fig. 2, *e* and *f*). HC supported high CAT activity regardless of which promoter was used (Fig. 3). Surprisingly, both constructs elicited strong CAT activity in CEF as well. In both cell types, the type X collagen promoter exhibited an activity equivalent to or higher than that of the HSV thymidine kinase promoter.

The sequence of the promoter and proximal flanking sequences within the 640-bp fragment are shown in Fig. 4. Comparison of this sequence with consensus regulatory sequence elements available from Genbank indicated that there are 85 possible regulatory sequence elements in this region. Of particular interest are enhancer consensus elements including the core enhancer sequence found in the rat α1(II) collagen promoter at −513 nt (Kohno et al., 1985); a consensus DNA binding site shared among homeobox proteins

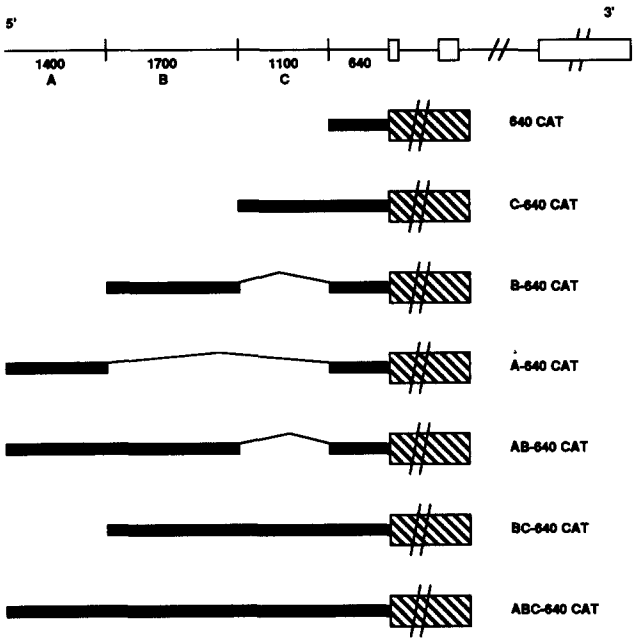

Figure 1. Collagen X promoter/CAT constructs. The collagen X gene is drawn schematically at the top of the figure. (□) Exons. *640* is the 640-bp fragment containing the transcription start and 558 bp of 5′ flanking sequence. *1,100*, *1,700*, and *1,400* refer to the DNA fragments located 5′ of 640 of sizes, 1,100, 1,700, and 1,400 bp. These fragments are also called fragments C, B, and A, respectively. Collagen X promoter/CAT constructs are depicted below the drawing of the collagen X gene. (■) Portions of the collagen X promoter and upstream regions which are present in the construct; black lines indicate portions of the upstream region which are deleted in the construct; (▨) the reporter gene, CAT.

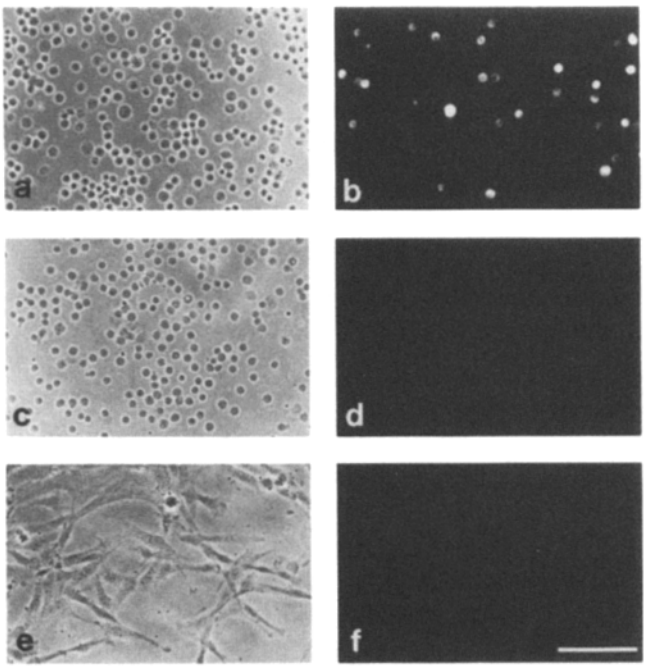

Figure 2. Phase (*a*, *c*, and *e*) and immunofluorescence (*b*, *d*, and *f*) micrographs of HC, IC, and CEF. Cells were grown in primary culture for ~7 d and were then processed for immunofluorescence using a collagen X antiserum. (*a* and *b*) HC; (*c* and *d*) IC; (*e* and *f*) CEF. Note that only hypertrophic chondrocytes produce detectable collagen X. Bar, 140 μm.

including yeast mating type MAT α2 and the human *oct 2* gene product, at −367 nt (Ko et al., 1988); two sequences which resemble retinoic acid–responsive elements at −346 and −280 nt (de The et al., 1990; Sucov et al., 1990; Vasios et al., 1989); a human immunoglobulin enhancer DNA binding domain at −211 nt (Maeda et al., 1987), which is likely to be a variant of the consensus sequence which is a potential binding site for the human oct 2 gene product; and an AP-2 consensus binding site at −126 nt (Imagawa et al., 1987).

Effects of Distal 5′ Flanking Sequences on the Ability of the Collagen X Promoter to Drive the Expression of CAT in HC, IC, and CEF

Because transfection of 640 CAT resulted in high CAT activ-

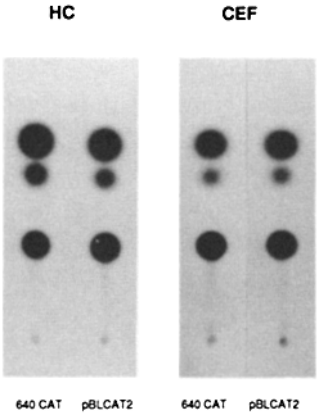

Figure 3. The effects of the 640-bp fragment on CAT activity in HC and CEF. This figure illustrates the effect of the 640-bp fragment on CAT activity in HC and CEF, and compares this activity with that of the positive control CAT vector, pBLCAT2. In both cell types, transfection with either 640 CAT or pBLCAT2 results in equivalent acetylation of CAT, as shown by the density of the upper two spots in each lane.

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                                                          -513
AAGCTTAGAA ATACAACTTT GGAGACAATC TCTGAATACA ATAACTGGTT -508

GGAAATAATC TTCGGATTCA TATACAACTC TCAAATTAAC ATCTTAACTG -458

ATTCTAAGCT CTCTATTTGC ACCTCAGTGA ACAACTACTC ACATAATCTC -408
                                                  -367
AGAGGAGTAC TCCTGAAAGT AACACCTCGA AGTCGAAAAT ATGTAATTCT -358
          -346
TTAGTTAAAA CGTTCACAGA AAAATCCAAA TGAAGCACCT AAAATTATAA -308
                              -280
ACCTTACTTA CAATTTTTTT CCATTTGTAA CCTTTCTCAT TTCTAAAAGC -258
                                                  -211
AGAAAGGATG CAAAAGTTTA GAAAATACGA AACTACAGGC AATACAATTT -208

TCATCAACAA GAAGAGGTCC AAGAGAAGAA GAAGAAATTA AAAGGCTTAA -158
                                  -126
CAGGACCTAA AATCCTACCC AAAATAGCAA CCCCCCATCC CTAAAACACA -108

CATATACACA CAGATAATTA CTGCTTACTG TCTTGGGGAG GAGCTTATGT  -58

TCAGCAGTCA CCAAACTAGG GTGAAAATCG TATAAATAGT CAAGGGTGAC   -8
   +1
GCTTGAAATC ATCAGCTTCT GCTCACTCAC CAGTGGCAGA AGAACTCCTC   43

ACCAGAGGAG GATTTGCCAC TCTACTGCCT TGCATTGGAG CT           85
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Figure 4. DNA sequence of the 640-bp fragment. The transcription start site is indicated by the number +1 above the A residue. The TATAA box is underlined. Putative enhancer elements discussed in the text are underscored by double underline and numbered at their 5′ residue. These elements are: an AP-2 site at −126 nt, an immunoglobulin enhancer site at −211 nt, two retinoic acid responsive elements at −280 and −346 nt, a homeobox protein consensus DNA binding site at −367 nt, and a core enhancer site (also found in the rat α1[II] collagen promoter) at −513 nt. Note that there is no proper CCAAT box within the first 100 bp 5′ of the transcription start site. These sequence data are available from EMBL/GenBank/DDBJ under accession number L11889.

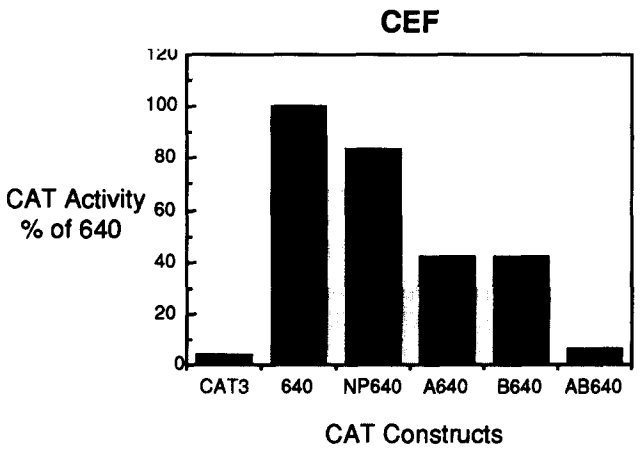

Figure 5. The effects of fragments A and B on the activity of 640 CAT in CEF. The activity of 640 CAT is represented as 100%. The presence of either fragment A or fragment B in the 640 CAT construct results in reductions of CAT activity of ~55–60%. The presence of fragments A and B, in the 640 CAT construct result in a >90% reduction in CAT activity when compared to the activity of 640 CAT. The presence of the unrelated NP fragment in the 640 CAT construct results in ~85% of the activity of 640 CAT; this is a negligible difference. The negative control CAT vector, pBLCAT3, demonstrates ~5% of the activity produced by 640 CAT. CAT activity was calculated as described in Materials and Methods. The range of values in this experiment was <4% of the average value shown.

ity in both HC and CEF, despite the fact that CEF do not transcribe the collagen X gene (Lu Valle et al., 1989), we performed further transfections with CEF using upstream sequences from the chick genomic clone PL10 (Lu Valle et al., 1988). A 3,100-bp fragment of upstream sequence (fragment AB in Fig. 1) was placed 5′ of 640 CAT to construct AB-640 CAT. In addition, fragment AB was digested with PstI to generate fragments A and B. These fragments were each ligated into the 640 CAT plasmid 5′ of the 640-bp fragment to generate A-640 CAT and B-640 CAT. CAT activity was assayed in CEF using these three constructs to examine their effects on the promoter activity of the 640-bp fragment. Fig. 5 shows that the presence of the AB fragment 5′ of 640 was associated with a 90–95% decrease in CAT activity in CEF when compared with the activity generated by the transfection with 640 CAT. In contrast, the presence of either the A fragment or the B fragment 5′ of the 640-bp fragment in CAT constructs resulted in a reduction of CAT activity of only 50–60% when compared with that of 640 CAT. pBLCAT3, the promoterless CAT plasmid, was used in this experiment as a negative control plasmid. The CAT activity generated by AB-640 CAT was approximately equivalent to the CAT activity generated by the negative control vector. In addition, when constructs containing a 3,300-bp fragment of unrelated DNA 5′ of 640, NP640 CAT, was transfected into CEF, there was no significant reduction in CAT activity.

Based on the results described above, different combinations of 5′ flanking fragments were inserted into 640 CAT to generate additional collagen X promoter–CAT constructs (ABC-640 CAT and BC-640 CAT; see Fig. 1). These constructs, as well as 640 CAT, AB-640 CAT, B-640 CAT, and C-640 CAT, were transfected into HC, IC, and CEF (Fig. 2, *a–f*). Transfection of these constructs into CEF and IC resulted in significant reductions in CAT activity when compared to 640 CAT (Fig. 6, *a* and *b*). The presence of fragment B or C (Fig. 1) in 640 CAT constructs produced a 75 and 50% reduction in CAT activity, respectively, in both CEF and IC (Fig. 6, *a* and *b*), while the presence of fragments AB, BC, or ABC reduced CAT activity by ~90% in both cell types. In contrast, transfections of the above constructs into HC resulted in high CAT activity, comparable to that resulting from transfection with 640 CAT. Transfections using B- or C-640 CAT resulted in activity equivalent to ~90% of that resulting from transfections with 640 CAT, while transfections using BC- AB-, or ABC-640 CAT resulted in 75–80% of the activity seen in cells transfected with 640 CAT (Fig. 6 *c*).

Discussion

Multiple Negative Elements Far Upstream of the Transcription Start Site Act in Conjunction with Elements Close to the Transcription Start to Confer Specific Expression on the Collagen X Gene

The results presented above indicate that the 4,200-bp 5′ flanking fragment of the chicken collagen X gene, when present 5′ of the 640-bp fragment, is able to restrict the expression of the reporter gene CAT to mature hypertrophic chondrocytes in culture. We show that the 640-bp fragment, which includes the transcription start site, promotes strong expression of CAT in IC, HC, and CEF. The addition of the 4,200-bp 5′ flanking fragment, ABC, dramatically reduces CAT activity driven by the collagen X promoter in non-collagen X-expressing cells but has no effect on the collagen X promoter-driven CAT activity in HC. Although the size of

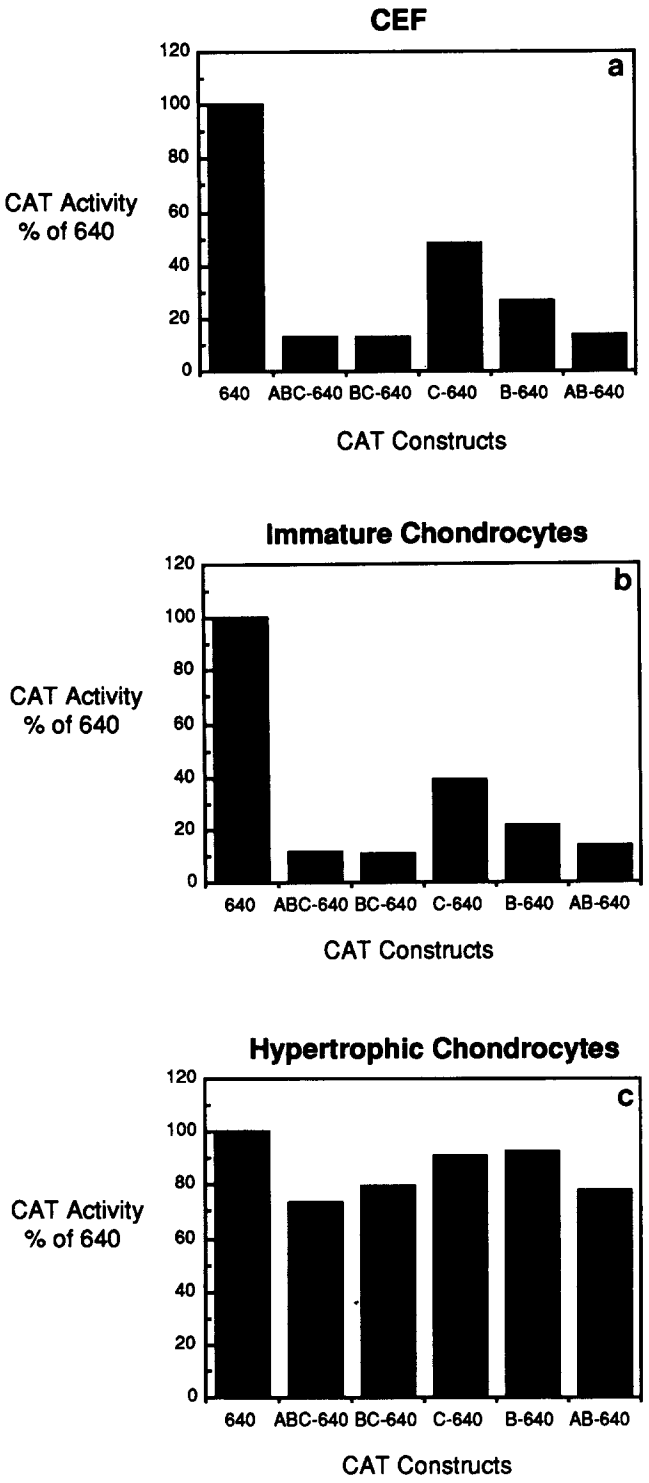

Figure 6. The effects of fragments A, B, and C on the activity of 640 CAT in CEF, IC, and HC. (*a*) The presence of fragments C or B in 640 CAT constructs resulted in a reduction of CAT activity to ~45 and 25% of the activity of 640 CAT in CEF, respectively. Combinations of fragments A, B, and C, (AB, BC, or ABC) in 640 CAT constructs reduced CAT activity to ~12% of the activity of 640 CAT. The range of values for each construct was <2% of the average value for each data point depicted here. (*b*) The reduction of CAT activity in response to the presence of fragments B or C in 640 CAT constructs in IC is ~40 and 23% of 640 CAT, respectively. Combinations of fragments A, B, and C, result in reductions equivalent to 10–15% of that resulting from 640 CAT. The range of values for each construct was <2% of the average value of each data point depicted here. (*c*) The same constructs used in transfections of CEF and IC were also used to transfect HC. The presence of 5′ flanking fragments A, B, and C singly and in combination in 640-bp constructs resulted in reductions in CAT activity ranging from less than 10% (fragments B or C) to 20–25% (fragments AB, BC, and ABC) of that of 640 CAT. The range of values for each construct was <5% of the average value of each data point depicted here.

the DNA fragment 5′ of the collagen X promoter fragment 640 did not affect promoter activity in HC, we tested this in CEF to eliminate the possibility that the large size of the 5′ flanking fragment, rather than silencer elements within the fragment, was responsible for inhibiting the activity of the promoter. The addition of a 3,300-bp fragment of unrelated DNA (part of the coding region of a nuclear pore complex protein, NP) 5′ of 640 in the CAT construct, had no affect on the strong promoter activity of the 640-bp fragment alone. Therefore we concluded that the effect of the collagen X gene fragment on the activity of the collagen X promoter in these constructs was due to specific sequence elements contained within the gene fragment, not because of a nonspecific effect due to its size. These data strongly suggest that the presence of the ABC fragment is required for the cell-specific CAT activity demonstrated herein, and therefore that this fragment may be instrumental in limiting collagen X gene expression to hypertrophic chondrocytes during endochondral ossification.

Negative regulatory elements have been identified in a variety of tissue-specific and developmentally regulated genes, such as the rat skeletal myosin heavy chain gene (Bouvagnet et al., 1987), the rat prolactin gene (Zhang et al., 1990), the mouse α1(I) collagen gene (Karsenty and de Crombrugghe, 1990; Ravazzolo et al., 1991), and the rat α1(II) collagen gene (Savagner et al., 1990). For example, in the α1(I) collagen gene, two negative factors and a positive factor interact with DNA sequences between bases −222 and −80, and the binding of one of the negative factors is inhibited by the presence of the positive factor (Karsenty and de Crombrugghe, 1990). In addition, the α1(I) collagen gene contains an upstream sequence between −361 and −339 which exhibits strong negative activity in fibroblasts but not other cell types (Ravazzolo et al., 1991). The rat α1(II) collagen gene contains two silencer elements located between −700 and −620, and −460 and −360; the silencers act together to significantly reduce promoter activity in fibroblasts, but not chondrocytes (Savagner et al., 1990). Since these two silencers confer tissue specificity on the type II collagen promoter, it is possible that the collagen II gene shares some regulatory mechanisms with the collagen X gene.

The three subfragments used independently in CAT constructs in the presence of the 640-bp fragment each act to partially repress the promoter activity of the 640-bp fragment in non-collagen X-expressing cells. Our data indicate that fragments A and B have a stronger effect than fragment C in this regard. In addition, fragment AB, BC, and ABC are more efficient at repressing CAT activity in 640 CAT than fragment A, B, or C alone. These results suggest that the repressor effect is additive, and in this regard is similar to the effect of the two silencers found in the 5′ flanking region of the collagen II gene (Savagner et al., 1990). Since the activity of the CAT constructs containing multiple subfragments is comparable with that of the background activity

demonstrated by the promoterless CAT construct, pBLCAT3, it is possible that the additive effect of these subfragments is sufficient to effectively silence the collagen X gene promoter in inappropriate cell types (i.e., IC and CEF). Our observation that silencing of this promoter requires several relatively weak elements spread diffusely over 4,200 bp of DNA, rather than one strong element, implies that this is a somewhat novel transcriptional regulatory mechanism. We are currently in the process of defining these regulatory elements and identifying their specific DNA binding proteins.

The potential problems surrounding the analysis of data generated from transient transfection assays are well known. Firstly, the DNA does not incorporate itself into the genome, therefore it is essentially naked, and unmodified. In contrast, genomic DNA contains chromatin, and is in a physical state such that its conformation may either allow or inhibit regulatory proteins from binding. In addition, various minor experimental variables can affect reporter gene activity resulting from transient transfections (Everett, 1988). The advantage is that it is an efficient and relatively inexpensive technique which is widely accepted. Nevertheless, promoter specificity should ideally be confirmed in an in vivo system where all possible regulatory influences are present, such as in a transgenic animal.

Recently, we have produced transgenic mice that carry a gene construct consisting of the chicken collagen X gene promoter and cell-specific repressor fragments fused to a full-length chicken collagen X cDNA containing an in-frame deletion in the triple helical-coding domain. The phenotype of these mice is characterized by a dramatic compression of the hypertrophic zone of skeletal elements undergoing endochondral ossification, as well as a reduction in bony trabeculae. Expression of the chicken collagen X transgene, detected by immunoreactivity to a mAb against chicken, but not mouse, collagen X, is limited to the abbreviated area of hypertrophic chondrocytes within the growth plates of the transgenic mice (Jacenko, O., P. Lu Valle, and B. R. Olsen, manuscript submitted for publication). These results strongly suggest that the negative regulatory elements in the upstream collagen X fragments are able to restrict expression of the gene to hypertrophic chondrocytes in vivo. They also imply that our data generated by reporter gene assays in cultured cells reflect the hypertrophic chondrocyte-specific mechanisms of collagen X gene expression operating in vivo. Future studies will involve examining the effect of inappropriate collagen X expression in transgenic mice by injection of a collagen X transgene driven by the nonspecific 640-bp portion of the chicken collagen X promoter, as well as transgenic experiments involving the inactivation of different areas of the endogenous mouse promoter.

The Collagen X Promoter Contains Strong Positive Element(s) within 550 bp Upstream of the Transcription Start Site

Sequences within the 640-bp fragment of the chicken collagen X gene, which includes the transcription start site, are sufficient to promote strong expression of the reporter gene CAT in CEF, IC, and HC relative to the expression generated by constructs containing repressor fragments. The expression of CAT in response to the presence of the 640-bp fragment and measured as acetylation activity is equivalent to or greater than CAT expression driven by the HSV thymidine kinase promoter in both HC and CEF. These data suggest that the non-cell specific collagen X gene promoter activity shown here is the result of nonspecific upstream activator sequences within the 640-bp fragment. The comparison of the sequence of this fragment with defined regulatory consensus sequences, as shown above, suggests that enhancer element(s) might be acting either singly or together to provide such promoter activity in the 640-bp fragment. Interestingly, this fragment has two features in common with the collagen II gene promoter: the presence in both of a core enhancer consensus sequence, and the absence in both of a proper CCAAT box within 100 bases of the transcription start site (Kohno et al., 1985). The presence of the homeobox protein DNA binding site consensus sequence at −367 nt may have significance, since homeobox proteins which potentially recognize this sequence (yeast MAT α2, and the human *oct-2* gene product) are gene regulatory proteins (Ko et al., 1988). The sequences resembling retinoic acid responsive elements located at −346 nt and −280 nt may be functional, since we have shown that retinoic acid treatment of cultured caudal embryonic sternal chondrocytes causes induction of collagen X gene expression (Oettinger and Pacifici, 1990; Pacifici et al., 1991*b*). The immunoglobulin enhancer binding motif (Maeda et al., 1987) found at −211 nt can act as a binding site for two enhancer proteins; one is found in all cell types, while the other is present only in lymphoid cells (Staudt et al., 1986). This site may also be a variant of the site at −367, since the *oct 2* gene product apparently encodes NF-A2, the lymphoid-specific protein (Ko et al., 1988). The consensus DNA binding site at −126 nt is recognized by the enhancer binding protein, AP-2. This protein can mediate gene transcription by means of signal transduction pathways involving either protein kinase C or cAMP-dependent protein kinase A (Imagawa et al., 1987). Currently, we are testing the possibility that these elements and/or other unidentified elements are active in either enhancing collagen X gene expression or mediating its repression by performing DNA binding studies using nuclear extracts with defined sequence elements within the 640-bp fragment.

Some collagen genes, including the α1(I) gene (Hartung et al., 1986), the α2(I) gene (Rossi and de Crombrugghe, 1987), α1(II) gene (Lovell-Badge, 1987; Horton et al., 1987), and the α1(IV) gene (Burbello et al., 1991), have been found to contain elements with enhancer activity in the first intron. Our results using constructs containing the first intron in addition to the 640-bp fragment do not demonstrate activity in significant excess of that seen with the 640-bp fragment alone (data not shown). This does not exclude the possibility that enhancer activity within the first intron of the collagen X gene is present, but not detectable by our in vitro assay system.

Taken together, the results of this investigation demonstrate that the 4,200-bp 5′ flanking fragment (fragment ABC) of the chicken collagen X gene, when present 5′ of the 640-bp promoter fragment in CAT constructs, limits CAT activity to mature hypertrophic chondrocytes. These data strongly suggest that the fragment ABC plays an important role in regulating the temporal and spatial expression of the collagen X gene. It is not yet clear whether a single consensus sequence, which would interact with the same DNA binding protein, is present multiple times within fragment ABC, or if several DNA binding proteins recognizing unique sequences spread diffusely throughout fragment ABC act together to confer cellular specificity on the collagen X gene

promoter. Preliminary analysis of consensus sequences within fragments A, B, and C suggest that the latter may be the case. Mobility shift assays using nuclear extracts from collagen X-expressing and -nonexpressing cells, as well as competition experiments involving binding between defined proteins and DNA sequence will provide more specific information about the mechanisms involved in the repression-mediated cell-specific transcription of the collagen X gene.

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