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, 1985a,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.
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 sternum 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., 1991a).

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) (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 x 10^6 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 days before transfection. CEF were obtained by digestion of tendon or skin from day 12-19 embryos with trypsin/collagenase as above. Resultant cells were plated at 1 x 10^6 cells/ml in the same medium as above.

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

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 pCH10 (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 polynucleotide 5' of the promoter, and pBLCAT3, a promoterless vector which contains a polynucleotide 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 polynucleotide 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 polynucleotide 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 polynucleotide 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 polynucleotide of the C-640 CAT vector. 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:

\[
\text{cpm acetylated}^{14}\text{C}^{1}\text{Clorhamphenicol} \times \frac{1}{\text{cpm}\text{ reacted}^{14}\text{C}^{1}\text{Clorhamphenicol}}
\]

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-

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1. Abbreviations used in this paper: CAT, chlamydomenial acetyl transferase; CEF, chick embryonic fibroblasts; HC, hypertrophic chondrocytes; HSV, herpes simplex virus; IC, immature chondrocytes.
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 α(II) collagen promoter at -513 nt (Kohno et al., 1985); a consensus DNA binding site shared among homeobox proteins 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 activi-
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 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.

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.
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 deletion in the triple helical-coding domain. The phenotype 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.

The Collagen X Promoter Contains Strong Positive Element(s) within 500 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.
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