Multiple Transcriptional Elements in the Avian Type X Collagen Gene

IDENTIFICATION OF Sp1 FAMILY PROTEINS AS REGULATORS FOR HIGH LEVEL EXPRESSION IN HYPERTROPHIC CHONDROCYTES*

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Fanxin Long‡, Gail E. Sonenshein§, and Thomas F. Linsenmayer‡‡

From the ‡Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111 and the §Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

During the cartilage-to-bone transition, participating chondrocytes eventually undergo hypertrophy and are replaced by bone and marrow. Type X collagen is synthesized by chondrocytes specifically when they become hypertrophic, and this specificity is primarily regulated at the level of transcription. Previously, we demonstrated that a proximal promoter region from nucleotide −562 to +86 contained cis-acting elements that directed high level expression of a reporter gene in a cell-specific manner (Long, F., and Linsenmayer, T. F. (1995) J. Biol. Chem. 270, 31310–31314). In the present study, we have further dissected this region by generating a series of constructs and examining their expression in hypertrophic versus nonhypertrophic chondrocytes. Several positive and negative elements have been delineated within the proximal promoter region to mediate the regulation of transcription in hypertrophic chondrocytes. Most notably, a sequence from nucleotide −139 to +5 was sufficient to direct high level expression in this cell type. Electrophoresis mobility shift assay and supershift experiments identified within this sequence two 10-base pair noncanonical binding sites for Sp1 proteins. Mutations within the Sp1 binding sites either diminished or abolished the expression driven by the sequence from −139 to +5. These results indicate that the Sp1 proteins mediate the cell-specific expression of type X collagen.

During endochondral bone formation, a notable event is the hypertrophy of chondrocytes prior to their removal and replacement by either bony tissue or marrow (1). A major product of hypertrophic chondrocytes is collagen type X (2–5). The chains of this homotrimeric molecule are encoded by a single gene, which becomes transcriptionally active following chondrocyte hypertrophy (6). This event occurs concomitant with a precipitous decrease in the production of certain other extracellular matrix molecules such as collagen types II and IX (6, 7), proteoglycan Lb (8), and cartilage matrix protein (9).

The tissue-specific expression of type X collagen, and several functional studies, suggest its potential importance for the development of endochondral bones. Individuals with Schmid metaphyseal chondrodysplasia have mutations in the type X collagen gene (10) that disrupt the assembly of the chains into a triple-helical molecule (11). In a transgenic study, major abnormalities in the cartilage growth plate were observed in mice carrying a chicken type X transgene with deletions (12). However, other investigators, using a gene knock-out technique (13, 14), reported only subtle phenotypic changes in a type X collagen-null mouse (14). Furthermore, type X collagen was shown to be associated with preexisting types IX/IX collagen fibrils by immunoelectron microscopy studies (15–17), and this was experimentally demonstrated in in situ diffusion studies (18). The diffusion studies also suggest that type X collagen may affect the biosynthesis of cartilage proteoglycans and alter the biochemical and physical properties of hypertrophic cartilage matrix (19).

The expression of type X collagen is controlled primarily at the level of transcription, as demonstrated by both in situ hybridization (6) and nuclear run-off experiments (20). We observed previously in transfection experiments, that a proximal promoter region that extended from 562 bp upstream to 86 bp downstream of the transcription start site conferred cell specificity for the expression of the gene (21).

In the present study, we have explored further the proximal promoter region and its neighboring sequences for their involvement in the tissue-specific regulation of the type X collagen gene. In transient transfection experiments with constructs containing serial deletions of the proximal promoter region, we found multiple regulatory elements that appear to contribute to the expression in hypertrophic chondrocytes. A detailed analysis focused on a sequence from −135 to +5, which conferred high level expression in this cell type, revealed two noncanonical Sp1 binding sites. The binding of Sp1 proteins to these sequences appears to be essential for high level expression in hypertrophic chondrocytes.

MATERIALS AND METHODS

Generation of CAT Constructs—The constructs pCAT I and pCAT II were as described previously (21). Constructs pCAT III through pCAT XII were generated as follows. The genomic inserts were obtained by PCR from the plasmid pFL2, which contained a 1.5-kilobase pair genomic fragment spanning the transcription start site of the type X collagen gene (21). To each corresponding genomic fragment, a HindIII site was added at the 5′ end and a SalI site at the 3′ end. The resultant constructs were purified with the QIAquick PCR Purification Kit (QIA-GEN Inc.) and subjected to HindIII and SalI digestion. The digested products were cloned into the HindIII and SalI sites of the vector pCAT-Basic (Promega). The resultant plasmids were sequenced (Life Technologies, Inc.) to confirm their identities. To generate constructs pCAT XIII, XIV, and XV, mutations were introduced into the sequence by PCR using primers containing these mutations. The resultant DNA was then cloned into pCAT-Basic as described above.

Cell Culture and Transfections—Primary cultures of hypertrophic

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‡ To whom correspondence should be addressed.

1 The abbreviations used are: bp, base pair(s); nt, nucleotide(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.
chondrocytes and nonhypertrophic chondrocytes were prepared from tibia and sternum of 14-day chick embryos, respectively, as described previously (21).

The transient transfections were done as described previously (21). Briefly, 1 μg of pSVβ-gal (Promega) was used as an internal control and cotransfected with 4 μg of constructs in 25 μg of Lipofectin (Life Technologies, Inc.). Cells were transfected for 5–8 h, allowed to recover in complete medium (Dulbecco’s modified Eagle’s medium from Life Technologies, Inc. supplemented with 10% calf serum from HyClone Laboratories) for 60 h, and the cells were harvested. In each experiment, transfections of each construct were done at least in triplicate. β-Galactosidase activity was measured according to Sambrook et al. (22) and expressed as the optical density of the substrate reaction at the wavelength of 420 nm (OD420). CAT (pg) was determined by enzyme-linked immunosorbent assay using an anti-CAT antibody (Boehringer Mannheim). Normalized levels of CAT expression were calculated as CAT protein to β-galactosidase (i.e. CAT/β-galactosidase). The mean value of CAT/β-galactosidase ratios from multiple transfections for each construct was taken as its CAT expression level in the experiment. Results presented in the paper are from two or more separate experiments.

**FIG. 1. A diagram of constructs used for transfections.** Shown for each construct is the insert cloned in CAT expression vector. Also shown is a partial organizational map of the type X collagen gene. The transcription start site is denoted by +1.

**TABLE I**

| pCAT (% ± S.D.) | I     | II    | III   | IV    | V     | VI    | VII   | X     | XI    | XII   |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                | 14.7 ± 0.6 | 14.7 ± 0.6 | 2.0   | 2.7 ± 0.6 | 2.7 ± 0.6 | 9.7 ± 0.6 | 4.0 ± 1.0 | 4.3 ± 0.6 | 26.3 ± 3.1 |

**RESULTS**

Multiple cis-Acting Elements Mediating Expression in Hypertrophic Chondrocytes—Previously, we showed that the prox-
mal promoter region, comprising 562 bp upstream and 86 bp downstream of the transcription start site (pCAT I, see Fig. 1), directs high level expression of a reporter gene in a cell-specific manner (21). To delineate the cis-acting elements within this region that are responsible for the regulation, we have now generated a series of constructs with nested deletions (see Fig. 1) and tested them by transient transfections. In nonhypertrophic chondrocytes, all constructs examined directed only a low level of expression. Specifically, the expression levels by these constructs in this type X-negative cell type were less than 30% of the expression elicited by pCAT I in hypertrophic chondrocytes (Table I). Examination of these constructs in hypertrophic chondrocytes, however, revealed a number of potential cis-acting elements, both positive and negative.

**Effects of the First Exon**—The first exon of the type X collagen gene appeared to contain a positive regulatory activity for transcription. The 86-bp transcribed sequence (nt +1 to +85, see Fig. 1) present in pCAT I represents a large portion of the first exon (26). The deletion construct pCAT III, however, contains only 5 bp of the transcribed region and thus lacks the bulk of the first exon (see Fig. 1). This construct elicited expression significantly lower than that by pCAT I (see Fig. 2), indicating the presence of a positive regulatory activity in the sequence from nt +6 to +85. Consistent with this, another construct that lacked the sequence, pCAT X, also directed considerably lower expression than pCAT XI, which contained it.

**Effects of an Upstream Sequence and the First Intron**—Previously, we reported that the 2.6-kilobase pair upstream sequence included in pCAT II did not affect cell specificity to any substantial degree. By comparing pCAT II and pCAT I within hypertrophic chondrocytes themselves, however, we found that expression by the former was consistently lower than that by the latter (see Fig. 2), suggesting that the 2.6-kilobase pair upstream sequence may actually repress expression.

The first intron of several collagen genes has been shown to contain regulatory activities responsible for tissue-specific expression (see “Discussion”). To test whether this is true for the type X collagen gene, we examined the expression by pCAT XII,

**Table II**

| Potential regulatory elements of type X collagen gene in hypertrophic chondrocytes |
|---------------------------------------------------------------|
| Approximate positions of elements | Regulatory activities |
|---------------------------------|---------------------|
| −3163 to −563                  | −                   |
| −562 to −493                   | +                   |
| −492 to −413                   | +                   |
| −412 to −292                   | −                   |
| −291 to −140                   | −                   |
| −139 to −115                   | +                   |
| −89 to −55                     | +                   |
| +6 to +85                      | +                   |

**Fig. 3. EMSA and competition assays.** The sequence from −139 to −55 was used as a probe, and its various regions were as competitors (Com1 through Com6) (A). Abbreviations in B are as follows: N, no nuclear extract; NC, no competitor; C1–C6, Com1–6; F, free probe. The arrows indicate the two major shifted bands.
a construct that contained the entire first intron in addition to the proximal promoter region (see Fig. 1). Our results showed that the expression level by this construct is similar to that by pCAT I (see Fig. 2), indicating that the first intron does not have any detectable effect on expression.

**Effects of the Sequence from nt −562 to −140**—To determine whether other regulatory elements might be present in the proximal promoter region, serial deletions were made from the 5’-end inward within the pCAT III insert (i.e., sequence from nt −562 to +5). This procedure resulted in the constructs pCAT IV through pCAT X (see Fig. 1). From the experiments using this set of constructs, a number of potentially interesting features of the promoter were uncovered.

A positive activity was found from nt −562 to −493, as the expression level elicited by pCAT IV was lower than that by pCAT III (see Fig. 2). Likewise, a second positive activity was located within the sequence from nt −492 to −413, as shown by the lower expression level by pCAT V compared with that by pCAT IV.

Negative regulatory activities were also found in the proximal promoter region. These were present from nt −412 to −292 and from nt −291 to −140, as demonstrated by an increase of expression by progressively shorter constructs, starting from pCAT V to pCAT VI to pCAT VII (see Fig. 2).

**Activation by Sequence from nt −139 to −55 in Hypertrophic Chondrocytes**—We next further dissected the sequence from −139 to −55, which appeared to contain a potent activity for high level expression in hypertrophic chondrocytes. Whereas pCAT X (containing the sequence from nt −54 to +5, see Fig. 1) elicited only a negligible level of expression, inclusion of the sequence from nt −139 to −55 in pCAT VII resulted in an 8–10-fold increase in CAT expression (Fig. 2). Further analyses
of the region from nt −139 to −55 revealed that sequences from nt −139 to −115, and from nt −89 to −55 are important for the high level expression. This is indicated by the results that the expression level dropped about 2-fold from pCAT VII to pCAT VIII and 3-fold from pCAT IX to pCAT X. Thus, by performing transient transfection experiments in hypertrophic chondrocytes, we have identified multiple regions with regulatory activities in the type X collagen gene. These results are summarized in Table II.

Identification of Core Sequences for Binding of Nuclear Proteins—To begin to examine the potential involvement of nuclear proteins in the regulation of expression in hypertrophic chondrocytes, EMSA was carried out using double-stranded DNA from nt −139 to −55 as a probe. We chose to examine this region because the transfections have shown that a construct containing this relatively short sequence, pCAT VII, elicited high level expression (about 70% of that by pCAT I) in hypertrophic chondrocytes (see above). Nuclear extracts prepared at different salt concentrations (0.8–1.6 M KCl) were examined, and all gave similar results (Fig. 3 and data not shown). Two major bands specific to the probe were detected (see Fig. 3B). To further dissect this region, a panel of oligonucleotides designed to cover the full length of the probe were used as competitors in EMSA (Fig. 3A). These experiments showed that DNA from nt −89 to −55 (Com4) competed for binding just as efficiently as the full-length cold probe (Com1), and that DNA from nt −139 to −115 (Com2) also competed for binding but to a somewhat lesser extent. DNA from nt −114 to −90 (Com3), from nt −120 to −94 (Com5), and from nt −99 to −75 (Com6), however, did not compete (see Fig. 3B). These results localized nucleotides important for the binding to the regions from nt −139 to −121 and from nt −75 to −55. Furthermore, both Com2 and Com4, when themselves used as probes in EMSA, exhibited two major bands (see Fig. 4, A and B) with mobilities similar to those produced by the full-length probe (Fig. 3B), confirming that they contribute to the binding of nuclear factors. These results are consistent with the finding from the transfection studies that nucleotides from nt −139 to −115 and from nt −89 to −55 are important for the high level expression conferred by the sequence from nt −139 to −55 (described above).

Sequence comparisons between the sequences from nt −139 to −121 and from nt −75 to −55 revealed two 10-bp nucleotide stretches of high similarity. These nucleotide stretches, located from −130 to −121 and from −74 to −65, are 5′-CCCATCCT-3' and 5′-TGGGAGGAG-3', respectively. We term them “core sequences” 1 (CS1) and 2 (CS2), respectively (see Fig. 4, A and B). To determine whether the core sequences are important for binding of nuclear factors, oligonucleotides containing mutations within these sequences were examined for competition in EMSA. As shown for probe Com2 in Fig. 4A, a mutation within CS1 (mutant 1) abolished its competition. Similarly for probe Com4, mutations within CS2 (mutants 2 and 3) abolished competition, whereas mutations in other regions had no effect (mutants 4 and 5) (Fig. 4B). These data indicate that CS1 and CS2 are essential for the sequence from −139 to −55 to bind nuclear proteins from hypertrophic chondrocytes.

To determine whether Com2 and Com4 bind the same proteins, these sequences were used to compete with each other in EMSA. Shown in Fig. 4A, Com4 competed with the probe Com2 and the competition was abolished by the mutation within CS2 (mutant 2). Likewise, Com2 competed with the probe Com4, and the competition was eliminated by a mutation within CS1 (mutant 1) (Fig. 4B). Thus, CS1 and CS2 appear to be involved in binding the same proteins.

Identification of the Core Sequences as Sp1 Binding Sites—A computer search identified 70% similarity between the core sequences and the consensus binding site for Sp1 and its related proteins. To determine whether it is Sp1 family proteins that are responsible for the binding to the core sequences in hypertrophic chondrocytes, we first performed additional competition assays using EMSA. In these assays, double-stranded oligonucleotides containing either a wild-type or a mutant Sp1 binding site were used as competitors for binding. As shown in Fig. 5A, in reactions using either Com2 or Com4 as a probe, the wild-type Sp1 oligonucleotide (lanes 4 and 10) disrupted formation of the two major complexes (arrows), whereas the mutant oligonucleotide Sp1', with a two-nucleotide alteration (see “Materials and Methods” for sequences), did not (lanes 5 and 11).
These results indicate that the Sp1 binding sequence specifically competes with both Com2 and Com4 for the binding.

To determine whether the core sequences bind Sp1, we performed supershift experiments using an antibody against this protein. As shown in Fig. 5A (lanes 6 and 12), the bottom band of the two main complexes (arrows) detected by Com2 and Com4 was supershifted by the Sp1 antibody. The upper band, however, does not appear to be shifted by the antibody. Thus, the lower band contains Sp1, whereas the upper band may contain a related family member. Overall, together with the competition data above, these results establish that both CS1 and CS2 bind Sp1.

A similar observation was also made with nuclear extracts from nonhypertrophic chondrocytes (Fig. 5B). Specifically, probes Com2 (lane 13) and Com4 (lane 18) both detected two major complexes that were competed out by the Sp1 sequence (lanes 15 and 20) but not by the mutant Sp1' oligonucleotide (lanes 16 and 21). Of the two complexes, the lower but not the upper band was also supershifted by the Sp1 antibody (lanes 17 and 22). Therefore, as in hypertrophic chondrocytes, Sp1 and its related proteins are also present and bind to the core sequences in nonhypertrophic chondrocytes. However, the ratio of the two complexes appears different between hypertrophic and nonhypertrophic chondrocytes. For instance, the bottom band formed by Com4 predominates over the upper band in nonhypertrophic chondrocytes (lane 18) whereas the two appear similar in intensity in hypertrophic chondrocytes (lane 8). These differences presumably reflect differential expression of the Sp1 family members in the different cell types, which could be important for cell-specific regulation of the type X collagen gene (see “Discussion”).

Effects of Mutations in Sp1 Binding Sites on Expression—To determine whether Sp1 family binding is functionally important for regulating expression of the type X collagen gene, we performed transfection experiments with mutant constructs. The mutations, in either CS1 or CS2 or both (Fig. 6A), were introduced into the construct pCAT VII and the effects of these alterations were examined for changes in the expression levels. As shown in Fig. 6B, a mutated CS1 (pCAT XIII) reduced expression by approximately 2-fold (compared with pCAT VII), and mutations in both CS1 and CS2 (pCAT XV) completely abolished expression (i.e. reduced it to the level of pCAT X). Likewise, a mutated CS2 alone (pCAT XIV) also eliminated expression. These data, when taken together with the results that these mutations abolish binding (see above), demonstrate that the binding of these Sp1 family proteins at both CS1 and

**Fig. 6. Effects of mutations within core sequences on CAT expression.** Nucleotides were mutated within either one or both of the sequences (A). Dotted lines indicate non-mutated nucleotides. Also shown is pCAT X, lacking the upstream region altogether. Shown in B are the effects of these mutations on expression. The expression by the wild type sequence (pCAT VII) is termed 100%. Results are expressed as mean ± S.D. of six transfections.
CS2 are important for high level expression in hypertrophic chondrocytes. Furthermore, the binding of Sp1 proteins at CS2 appears to be indispensable.

DISCUSSION

In the present study, we have uncovered a number of potential cis-acting elements involved in the transcriptional control of the chicken type X collagen gene. Most notably, an 85-bp sequence from nt −139 to −55 contained a potent positive regulatory activity and this sequence, in conjunction with a short fragment containing the TATA box (nt −54 to +5), was sufficient to direct high level expression in a cell-specific manner. Detailed analysis of this region revealed two noncanonical Sp1 binding sites, whose binding to Sp1 family proteins appeared to be critical for high level expression of the gene in hypertrophic chondrocytes.

The present study suggests that the Sp1 family proteins mediate the expression of type X collagen in a cell-specific manner. Similar cell-specific regulation by these factors has also been reported for other matrix proteins, including those of collagens and elastin (27–30). Although the exact mechanism for this is unclear at present, several scenarios can be postulated. One is based on information showing that not all members of the family produce the same regulation. For instance, it is known that Sp3 functions as an inhibitor, whereas Sp1 is an activator (29, 31). Thus it is conceivable that, in chondrocytes at different stages of maturation, regulation is achieved by the relative level or availability of these factors. This is of particular interest in light of our gel shift data, which showed differential relative intensities for the bands of Sp1 family proteins in hypertrophic chondrocytes.

Alternatively, other protein-DNA interactions in the promoter may influence the function of Sp1 proteins in nonhypertrophic chondrocytes. In support of this possibility, our preliminary data showed that when an 85-bp fragment (−139 to −55) containing the Sp1 sites and their neighboring sequence was used as an EMSA probe, three additional bands exclusive to nonhypertrophic chondrocytes were detected, in addition to the two major Sp1 complexes (data not shown). Two of the three bands appear to represent higher order Sp1 complexes. The third band, however, seems to be a separate entity, since its formation occurs more efficiently in the absence of Sp1 binding. These observations, when coupled with the result that this 85-bp sequence conferred cell specificity in transfections, suggest a possible inhibitory mechanism in the nonhypertrophic chondrocytes.

Nonetheless, Sp1 appears to be critical for the high level expression of type X collagen in hypertrophic chondrocytes. Nucleotide comparisons of the type X collagen gene across several species revealed that the Sp1 binding site CS2 identified in this study is greatly conserved throughout evolution (see Table III). For instance, the promoter for the human gene contains an element that is 90% identical with the chicken CS2, despite the fact that the overall similarity between the two species from nt −100 to −1 is only about 60%.

Of the two Sp1 binding sites, CS2 appears to have a higher affinity. This is suggested both by the competition analyses in which Com4 (containing CS2) competed better than Com2 (containing CS1) (Fig. 3B), and by the mutation study where probe 2 (with a mutated CS1) bound Sp1 more efficiently than probe 1 (with a mutated CS2) (data not shown). This differential binding affinity is consistent with the greater reduction of expression in transfections by the mutation in CS2 versus that in CS1 (see Fig. 6B). The mechanism(s) by which CS1 and CS2 combinatorially regulate expression of the gene remain to be investigated. Nonetheless, CS2 appears to play a pivotal role for activation of the gene. Activation by CS1 is not only synergistic with that by CS2 but also seems to be dependent upon the presence of CS2.

The present study also revealed several other potential regulatory regions in the promoter of the type X collagen gene. For instance, the sequence from nt −562 to −413 appeared to have a positive regulatory activity. Conversely, the fragment from nt −3163 to −563 and that from nt −412 to −140 seemed to repress expression in hypertrophic chondrocytes. Further studies, however, are required to elucidate the regulation in these regions. Taken together, the results from this study suggest that multiple cis-acting regulatory elements exist within the proximal promoter region of the chicken type X collagen gene and that Sp1 family proteins mediate signals promoting the cell-specific expression of the type X collagen gene.

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| TABLE III | Conservation of a Sp1 binding site (CS2) across species |
| Bold letters denote nucleotides conserved with those in the chicken. |
| Species | Sequence | Position | Reference |
| Human | 5’-AGGGGAGGAGG-3’ | nt −80 to −71 | (36) |
| Bovine | 5’-GGGGAGGAGG-3’ | nt −82 to −73 | (36) |
| Mouse | 5’-AGGGGAGGAGG-3’ | nt −85 to −76 | (37) |
| Chicken | 5’-GGGGGAGGAGG-3’ | nt −74 to −65 | (26) |
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