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Proximal DNA Elements Mediate Repressor Activity Conferred by the Distal Portion of the Chicken Collagen X Promoter

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Abstract Collagen X is expressed specifically in hypertrophic chondrocytes within cartilage that is undergoing endochondral ossification. The chicken collagen X gene is transcriptionally regulated, and under the control of multiple cis elements within the distal promoter region (−4,442 to −558 base pairs from the transcription start) as well as the proximal region (−558 to +1). Our previous data (LuValle et al., [1993] J. Cell Biol. 121:1173–1179) demonstrated that the proximal sequence directed high reporter gene activity in the three cell types tested (hypertrophic chondrocytes, immature chondrocytes, and fibroblasts), while distal elements acted in an additive manner to repress the effects of the proximal sequence on reporter gene activity in non-collagen X expressing cells only (immature chondrocytes and fibroblasts). We show here that elements within the proximal sequence (nucleotides −557 to −513) are necessary for the cell-specific expression of type X collagen by hypertrophic chondrocytes. These elements bind to proteins of 100 kDa in all three cell types, and 47 kDa in non-collagen X expressing cells. Reporter gene activity in hypertrophic chondrocytes is reduced to the levels seen in non-collagen X-expressing cells in the absence of these elements. J. Cell. Biochem. 70:507–516, 1998.

Key words: type X collagen; transcription

The defining anatomical feature of developing endochondral bone is the area of cartilage cell differentiation, where chondrocytes undergo a series of phenotypic changes from quiescent, to rapidly proliferating, to post-mitotic hypertrophic chondrocytes that finally undergo apoptosis (or may transdifferentiate to osteoblast cells) [Gentili et al., 1993; Kirsch et al., 1992; Moskalewski and Malejczyk, 1989; Roach et al., 1995]. These cellular changes are associated with changes in the cartilage extracellular matrix (ECM) including synthesis, calcification, degradation, and replacement by bone and marrow. Chondrocyte hypertrophy is specifically marked by the synthesis of collagen X [Jacenko et al., 1991].

Collagen X is secreted by hypertrophic chondrocytes as a homotrimer. It assembles into a pericellular hexagonal lattice-like configuration in vitro [Kwan et al., 1991] and it also migrates into the interterritorial matrix where it interacts with cartilage fibrils [Chen et al., 1990]. The transcriptional activation of collagen X in hypertrophic chondrocytes [LuValle et al., 1989] coincides with decreases in collagen II [LuValle et al., 1992] and IX [Linsenmayer et al., 1991] mRNA. The protein represents approximately 45% of the total collagen secreted by hypertrophic chondrocytes [Reginato et al., 1986].

The function of collagen X has been under investigation for some time. Deletions in the human collagen X coding sequence result in autosomal dominant Schmid chondrodysplasia [Warman et al., 1993]. The phenotype of a mouse that overexpresses a truncated chicken collagen X gene in hypertrophic chondrocytes (under the control of the chicken collagen X promoter (CCXP) is consistent with that seen in Schmid chondrodysplasia [Jacenko et al., 1993]. Two independent studies involving the production of collagen X-null mice show conflicting results. While results from the first study did not reveal an abnormal phenotype [Rosati et al., 1994], the more recent study [Kwan et al.,

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1997] describes a phenotype with characteristics in common with that of Schmid chondrodysplasia. The data from the more recent knockout mouse as well as the transgenic and human genetic data suggest that collagen X is necessary for proper endochondral bone formation.

Collagen X is unique among collagens in that it is temporally and spatially limited to hypertrophic chondrocytes, regardless of whether these cells are found in growth plate, osteoarthritic cartilage, or fracture callus [Jacenko et al., 1991]. In vivo, initiation of collagen X transcription is coincident with chondrocyte hypertrophy [LuValle et al., 1989, 1992]. Our previous data suggested that the initiation of hypertrophic chondrocyte-specific collagen X gene transcription involved (1) inhibition of collagen X gene expression in non-collagen X expressing cells (immature chondrocytes and fibroblasts) by multiple, additive repressor elements located in the distal promoter region of the gene, and (2) activation of positive effectors in the proximal promoter region in hypertrophic chondrocytes, as demonstrated by reporter gene assays in primary chicken chondrocytes isolated from embryonic sterna [LuValle et al., 1993]. In the present study, we have generated data indicating that DNA elements within the most 5' 44 bp of the proximal promoter region block upstream inhibition of collagen X expression in hypertrophic chondrocytes. We propose a simplified mechanism whereby proteins of 100 and 47 kDa, identified by Southwestern analyses, bind to these DNA elements and mediate hypertrophic chondrocyte-specific transcription of the collagen X gene.

EXPERIMENTAL PROCEDURES

Recombinant Reporter Plasmids

The salient features of the constructs used in this study are illustrated in Figure 1. All restriction enzymes and linkers were from New England Biolabs (Beverly, MA). The PRLnull plasmids (Promega, Madison, WI) utilized the Renilla firefly luciferase gene under the transcriptional control of the chicken collagen X promoter sequences. The 3' Sac I site in the 640 bp proximal CCXP (nt −558 to +82) was removed and replaced with a Sal I site. The fragment was subsequently cloned into pBluescriptIIKS (PBSIIKS, Promega). It was then liberated from PBSIIKS with Hind III and Sal I, and subcloned into PRLnull to generate 640 LUC. 483(Δ157) LUC was created by restricting the proximal CCXP with Hind III and Csp61 to remove the most distal 157 bp. The Csp61 site was then blunt-ended and religated to the Hind III site in the polylinker of PRLnull using HindIII linkers (New England Biolabs). 527(Δ113) LUC was generated by blunt-ending the Csp61 site as above, digesting with Hind III to remove the 157 bp fragment, and ligating a double-stranded synthetic 44 bp oligonucleotide (with the same sequence as the most distal 44 bp in 640 LUC) with a 3′ blunt end and a 5′ Hind III overhang into the blunted Csp61 site and the Hind III site within the PRLnull polylinker region. The 596(Δ44) LUC construct was created by first removing the full-length proximal CCXP from PBSIIKS with Hind III and Sal I, as above. This 640 bp fragment was digested with BsrI to remove the most 5′ 44 bp. After ligating the Sal I site back into the PRLnull polylinker region, the 5′ BsrI site was blunt ended, Hind III linkers were added, and the 5′ end was religated into the Hind III site of the PRLnull polylinker region. ABC640LUC was generated by removing the distal CCXP (−4443 to −558) from the plasmid PL10 [LuValle et al., 1988], replacing the 5' EcoRI site with a SpeI site, and subcloning it into the SpeI and Hind III sites adjacent to and upstream of the proximal CCXP. ABCΔ44 LUC, ABCΔ113 LUC, and ABCΔ157 LUC were all generated in a similar manner.

Cell Culture, Transfections, and Reporter Assays

Chondrocytes and fibroblasts were isolated from embryonic sterna and toe tendons, respectively, as described previously [LuValle et al., 1993]. Chondrocytes from upper sterna (hypertrophic chondrocytes) were grown in suspension in DMEM (high glucose) with 10% FCS (Hyclone Labs, Logan, UT) for 1 week prior to plating for assay in order to optimize the number of cells expressing collagen X. Immature chondrocytes and embryonic fibroblasts were plated for assay directly in 6-well culture dishes (35 mm wells) at a density of 1–5 x 10^5 cells per well and cultured to 50–60% confluency (about 2 days) prior to transfection. Hypertrophic chondrocytes were cultured for 1 week prior to transfection. All cells were treated with hyaluronidase (Sigma, St. Louis, MO, 4 μg/ml of medium) 4 h prior to transfection in order to disrupt the extracellular matrix to allow for optimized transfection as described previously [LuValle et al., 1993]. Two micrograms of each construct

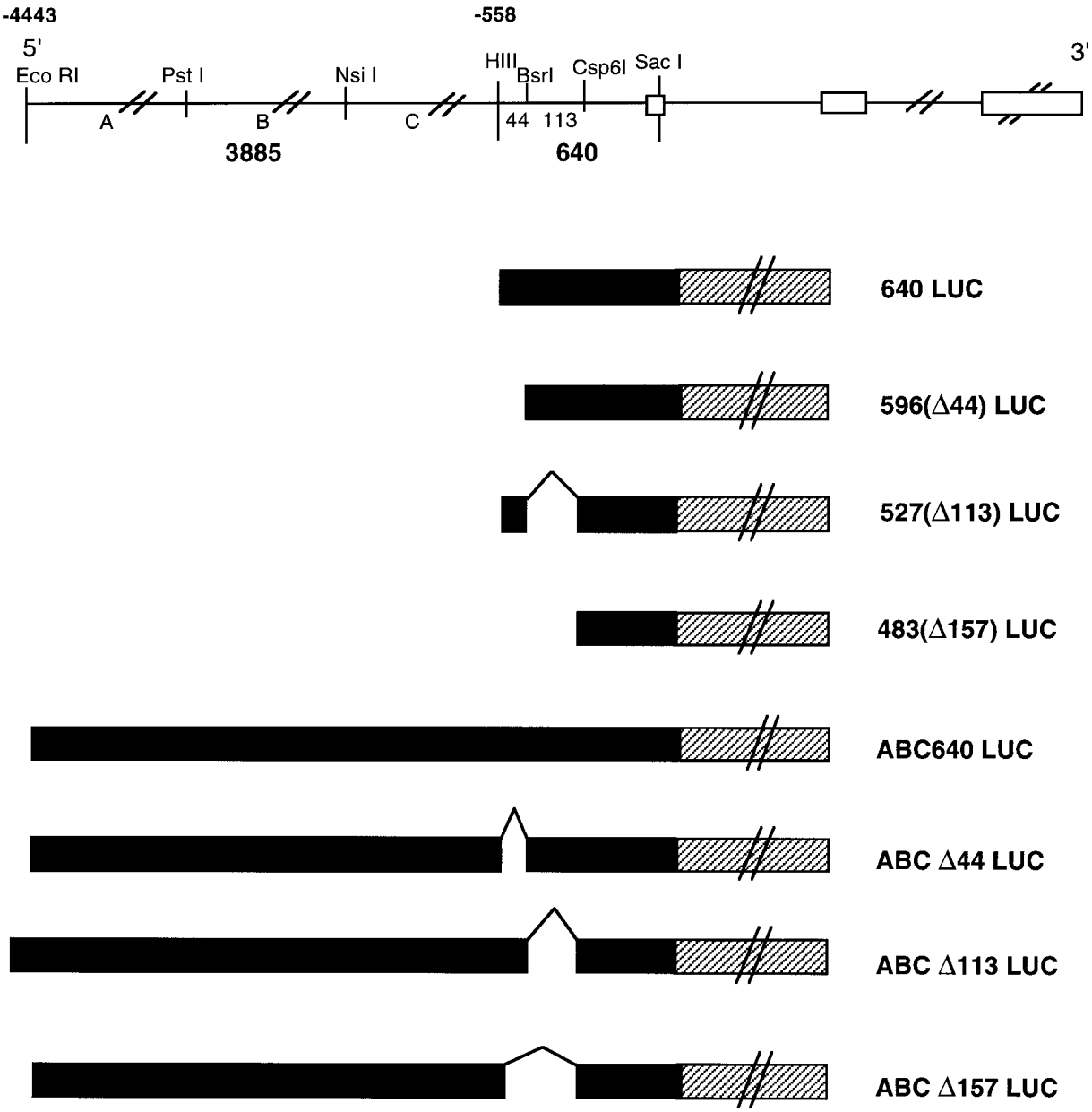


Fig. 1. Luciferase reporter constructs. A map of the collagen X gene is shown. The 5′ 3,885 bp region is the distal CCXP. The 640 bp region is the proximal CCXP. The hatched rectangular areas represent the luciferase reporter plasmid, while the black areas represent the portions of the chicken collagen X promoter present in each construct.

was co-precipitated with 1 µg of pGL-2 control vector (Promega) and resuspended in 25 µl Hepes buffer, pH 7.4. This was incubated with a 1:20 dilution of DOTAP transfection reagent (Boerhinger Mannheim, Indianapolis, IN) for 15 min. The cells were washed three times with DMEM and antibiotics in the absence of serum prior to the addition of the DNA-DOTAP mixture to the cells in 2 ml of DMEM without serum and incubation for 5–8 h at 37° C in the presence of 5% carbon dioxide, 95% air. The medium was then replaced with complete medium, and the cells were incubated for another 48 h. Luciferase activity was measured with the Dual-Luciferase Reporter Assay kit (Promega). Cells were washed twice with PBS and lysed according to manufacturers' instructions. Firefly luciferase cDNA under the control of the SV40 promoter was used to normalize Renilla luciferase activity within each dish. Activity was measured on a Turner TD-20e Luminometer (Turner Designs, Sunnyvale, CA).

Preparation of Nuclear Extracts

Cells were dissected from chicken embryos as described [LuValle et al., 1993]. They were pelleted, washed twice with ice-cold PBS, and counted with a hemocytometer. Each cell type was resuspended in 40 volumes of ice cold lysis buffer (10 mM Tris, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$) containing a protease inhibitor cocktail (0.5 mM each PMSF and dithiothreitol, and 1 µg/ml each of chymostatin, leupeptin, and pepstatin A, present in all buffers used for this procedure) and allowed to swell for 10 min on ice. Cells were then pelleted for 5 min at 1,500 rpm and resuspended in lysis buffer at 1 ml/5 x 10^7 cells. An equal volume of ice-cold NP-40 lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) was added, and the resulting cell suspension was homogenized in dounce homogenizers using the loose (B) pestle. The homogenate was transferred to round-bottom centrifuge tubes and nuclei were pelleted at 2,000 rpm for 10 min. After removal of the supernatant, the pellet was spun again at 25,000*g* for 20 min. The remainder of the protocol is as described in Dignum et al. [Dignam et al., 1983] using either 0.4 M or 0.6 M NaCl in the extraction buffer. The resulting supernatant in buffer containing 20 mM Hepes pH 7.9, 0.1 M KCl, 0.2 mM EDTA, and 20% glycerol (buffer D) was frozen in 100 µg aliquots at −80° C after measuring protein content using the Bradford protein assay [Bradford, 1976].

Electrophoretic Mobility Shift Assays

DNA fragments were labeled by fill-in reaction using the Klenow fragment of DNA polymerase I. Nuclear extract (10–15 µg) was incubated with 10^4 cpm of labeled probe in the presence of 2 µg poly(dI-dC)·poly(dI-dC) (Sigma), 300 µg/ml BSA, 80 mM NaCl, 120 mM KCl, 0.5 mM MgCl, 0.2 mM ZnCl, in a final volume of 15 µl for 15 min at 30°C. Competition experiments involved a 30°C 15-min pre-incubation of nuclear extracts in the presence of 100 M excess unlabeled DNA probe prior to the addition of labeled probe [Chodish, 1988]. After incubation, the binding reaction was electrophoresed in a 4% native polyacrylamide gel at 35 mA for 2 h in 0.5 X TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8). The gel was then dried and autoradiographed using X-OMAT AR Kodak film.

Southwestern Analyses

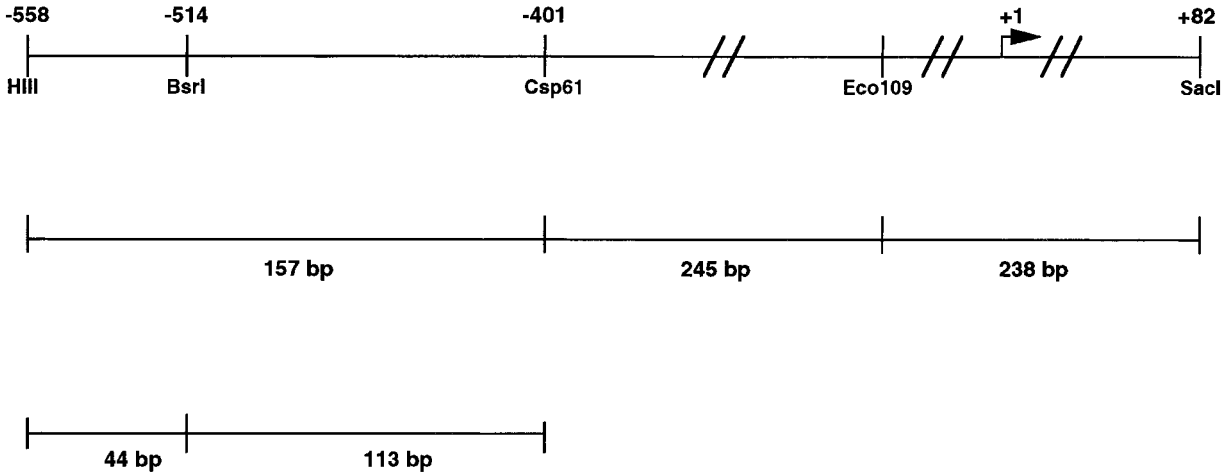
Southwestern blotting was performed essentially as described by Farrell et al. [1990]. Nuclear proteins (100 µg) from all three cell types were boiled in the presence of an equal volume of SDS sample buffer (22) for 5 min and then placed on ice. The samples were separated by SDS PAGE using a 10% gel at 25 mA for 2 h in electrophoresis buffer (50 mM Tris, 400 mM glycine, 0.1% SDS). The gel was incubated in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) for 30 min to allow renaturation of proteins prior to electroblot transfer onto a nitrocellulose membrane using a Bio-Rad (Richmond, CA) transfer cell overnight at 25 mA. 10^6 cpm/ml (1 µg) of labeled probe (44 bp, labeled with ^{32}P-dCTP and dATP by fill-in reaction with Klenow enzyme) was used in the hybridization. The blot was exposed to X-OMAT AR film overnight. Control blots were pre-incubated with twofold quantities of unlabeled DNA for 1 h prior to adding labeled probe.

RESULTS

Identification of Sequences Within the Proximal Promoter Region That Affect CCXP Activity

The proximal promoter region of the CCXP is defined by the restriction endonuclease site Hind III and Sac I, and includes 558 bp of sequence 5' of the transcription start site as well as part of the first exon (Figs. 1 and 2). The sequence from −558 to +1 contains several potentially interesting consensus transcription factor binding sites [LuValle et al., 1993] However, we were specifically interested in identifying sequence elements that might moderate the repressor activity of the distal CCXP. We divided the proximal CCXP into 3 sections with convenient restriction endonucleases. The sequence of the most 5′ fragment, a 157 bp fragment delineated by Hind III and Csp61 restriction endonuclease recognition sites (at −558 and −401 nt, respectively), is shown in Figure 2. We used this fragment for electrophoretic mobility shift assays with nuclear extracts from hypertrophic chondrocytes as well as immature chondrocytes, and were able to identify mobility shift patterns that were specific only for immature chondrocytes (data not shown).

Deletions of this fragment were used in reporter constructs to assess their effect on the repressor activity previously identified in the



157 bp sequence

AAGCTTAGAAATACAACTTTGGAGACAATCTCTGAATACAATAACTGGTTGGAAATAATCTTCGGA
TTCATATACAACTCTCAAATTAACATCTTAACTGATTCTAAGCTCTCTATTTGCACCTCAGTGAACA
ACTACTCACATAATCTCAGAGGA

Fig. 2. The restriction map and sequence of the 157 bp fragment. The 157 bp fragment is located at nt −558 to −401 at the 5′ end of the 640 bp proximal promoter region of the collagen X gene. It is delineated by a Hind III (HIII) site at its 5′ end and a Csp61 site at its 3′ end. It is bisected at nt −514 by a Bsr I site to yield a 5′ 44 bp fragment and a 3′ 113 bp fragment. The 44 nt portion of the 157 nt sequence is underlined.

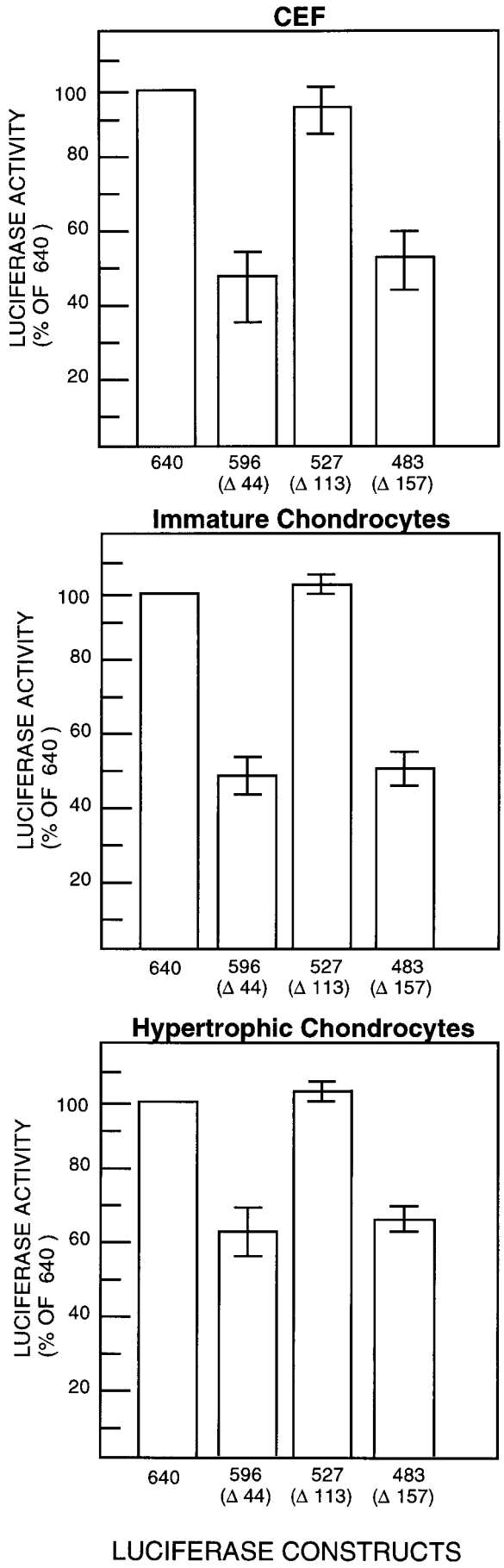
3,885 bp distal promoter region termed ABC [LuValle et al., 1993]. Figure 1 is a map of the constructs used to analyse luciferase activity under the control of the CCXP in hypertrophic chondrocytes, immature chondrocytes, and embryonic fibroblasts. Essentially, the 157 bp fragment (sequence shown in Fig. 2) was either deleted completely (Δ157) or partially (Δ44 or Δ113) as described in Experimental Procedures, and then used in the context of the remainder of the 640 bp fragment with or without the distal 3885 bp to drive luciferase activity in the three cell types.

Figure 3 shows the results of luciferase assays performed with the proximal CCXP deletion constructs in the absence of the distal CCXP. Luciferase activity was reduced by approximately 40–50% in all three cell types as a result of deleting nt −558 to −514 (Δ44) or −558 to −401 (Δ157). Deletion of nt −514 to −401 (Δ113) had no effect on luciferase activity when compared to the construct containing the entire proximal CCXP (640).

Figure 4 shows the results of luciferase assays performed with the same proximal CCXP deletion constructs used in Figure 3, in the presence of the distal CCXP. The ABC640 construct repressed luciferase activity by approximately 90% in immature chondrocytes and embryonic fibroblasts (CEF), but had a relatively insignificant effect in hypertrophic chondrocytes, as shown previously [LuValle et al., 1993]. The Δ113 deletion had no effect on this pattern of activity in any of the cell types. The Δ44 and Δ157 deletions had no effect on the repression of luciferase activity in non-collagen X expressing cells, but caused a dramatic reduction in activity (approximately 85%) in hypertrophic chondrocytes.

DNA-Protein Interactions Within −558 to −514 of the Proximal CCXP

Figure 5 shows the results of Southwestern analyses of the interactions between the 44 bp subfragment (nt −558 to −514) and nuclear proteins extracted from hypertrophic chondrocytes, immature chondrocytes, and embryonic fibroblasts. The data show that the labeled fragment interacts with protein of approximately



100 kDa in extracts from all three cell types, and with protein of approximately 47 kDa in extracts from non-collagen X-expressing cell types only. Control experiments using twofold excess unlabeled probe to preincubate blots prior to hybridization with labeled probe resulted in no signal on autoradiograph, indicating that interactions between the 44 bp fragment and nuclear proteins were specific (data not shown).

DISCUSSION

DNA Elements Within −558 to −514 in the Proximal Region of the CCXP Are Required for Optimal Expression of Collagen X in Hypertrophic Chondrocytes

We have previously demonstrated that the distal 3,885 bp of the CCXP (from −4,442 to −558 bp) confers repression on CAT reporter activity under the control of the proximal CCXP (−558 to +82 bp) in non-collagen X-expressing cells (immature chondrocytes and fibroblasts), and that this same distal regulatory region has no significant effect on reporter activity directed by the proximal promoter in collagen X-expressing (hypertrophic) chondrocytes [LuValle et al., 1993]. We show here that this distal repressor activity in non-collagen X expressing cells is unaffected by deletions within −558 to −401 nt from the transcription start, as indicated by data from immature chondrocytes and fibroblasts showing approximately 90% reduction in reporter activity in the presence of the distal 3,884 bp regardless of deletions within the proximal promoter region. However, proximal DNA elements(s) within −558 to −514 nt appear to be involved in abrogating the effects of the negative elements found within the distal 3,885 bp in hypertrophic chondrocytes, as determined by the substantial decrease in reporter activity (approximately 85%) when the sequence from −558 to −514 is absent. This "de-repression" allows optimal expression of reporter activity in collagen X-expressing (hypertrophic) chondrocytes. If the sequence

Fig. 3. The effect of deletions in the proximal CCXP on luciferase reporter gene activity in the absence of the distal 3,885 bp. Reporter plasmids were constructed and assays were performed as described in Experimental Procedures. Renilla luciferase activity was measured after transfection of CEF, immature chondrocytes, and hypertrophic chondrocytes. Data was normalized by cotransfection with firefly luciferase reporter plasmid. Each data point represents the average and standard error of a minimum of six replicates. Each graph represents a representative experiment that was repeated at least twice.

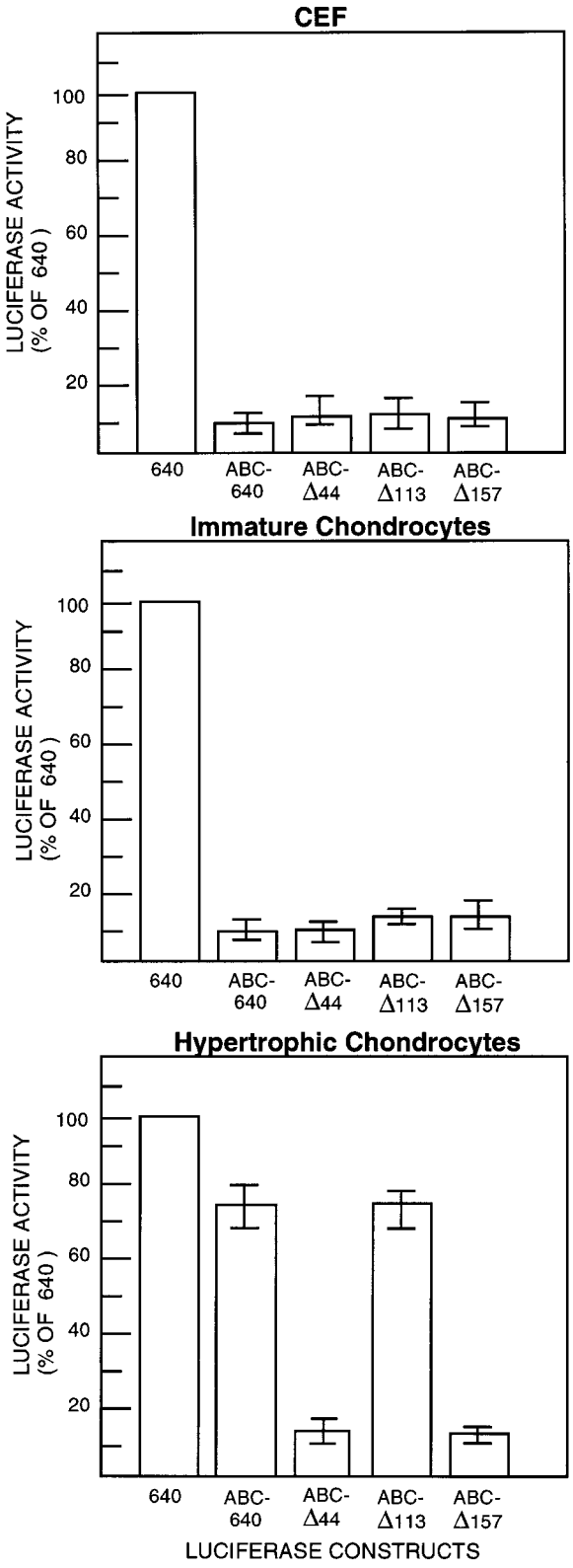


Fig. 4. The effect of deletions in the 157 bp fragment of the CCXP on luciferase reporter activity in the presence of the distal 3885 bp. See legend for Figure 3.

within −558 to −514 contained an enhancer, rather than a "de-repressor," one would expect that deletion of the sequence in reporter constructs in the absence of the influence of the distal 3,385 bp would result in a similar strong decrease in reporter activity. However, this deletion results in a relatively mild decrease in activity of 40 to 50% in all three cell types. In addition, when the distal CCXP is present, the "de-repressor" sequence appears to be active only in hypertrophic chondrocytes, suggesting that the control element(s) within that sequence are able to negate the overall repressor effect of the distal CCXP.

De-Repression of Collagen X Transcription in Hypertrophic Chondrocytes Is Achieved by DNA-Protein Interactions

The 44 bp sequence located between nt −558 and −514 contains three putative transcription factor binding sites according to TFSEARCH results (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). A heatshock factor binding site found in both yeast and Drosophila (AGAAA) is located at nt −522 [Fernandes et al., 1994]. The yeast alcohol dehydrogenase gene regulatory factor consensus DNA binding site (TTGGAGA) is located at nt −538 [Cheng et al., 1994]. A consensus binding site with 85% homology to the site that interacts with the product of the chicken *cdxA* homeobox gene (ATTTATA) is located at nt −553 [Margalit et al., 1993]. The predicted size of the translated product of the cdxA gene is approximately 27 kDa, which is significantly smaller than the products identified by Southwestern analyses.

The results of the Southwestern analyses suggest that protein(s) of 100 kDa interact with the 44 bp subfragment in both collagen X- and non-collagen X-expressing cells, and that protein(s) of 47 kDa interact with this subfragment only in non-collagen X-expressing cells. One interpretation of these data is that the key repressor proteins that interact with DNA elements in the upstream region (−4,442 to − 558 nt) remain bound to the DNA in hypertrophic chondrocytes, but cannot confer repression on the promoter because hypertrophic chondrocytes do not produce a regulatory protein that blocks collagen X transcription (in this case, the 47 kDa protein species identified by Southwestern analyses). This interpretation is supported

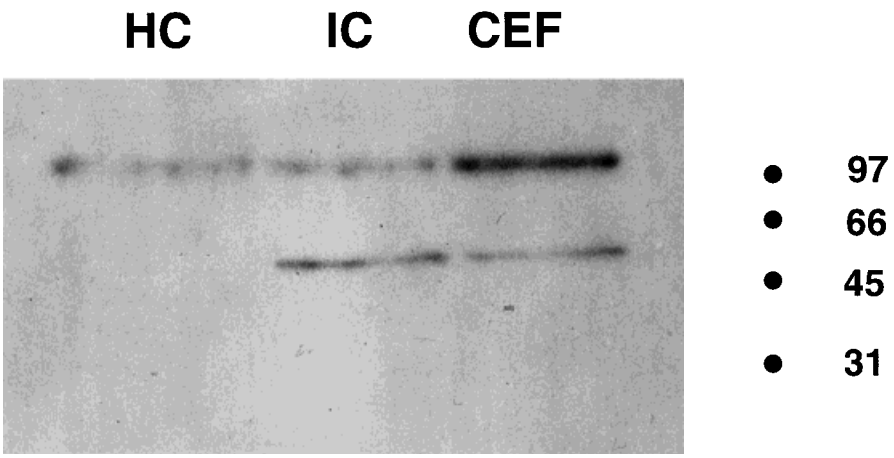


Fig. 5. Southwestern analyses of the 44 bp subfragment. Nuclear extracts from hypertrophic chondrocytes (HC), immature chondrocytes (IC), and embryonic fibroblasts (CEF) were subjected to SDS PAGE, blotted onto nitrocellulose, and hybridized to radiolabeled 44 bp fragment. To the right are molecular mass values in kDa.

by the results of the reporter activity assays presented here that indicate repression of luciferase activity in hypertrophic chondrocytes in the absence of the putative de-repressor elements. Our data suggests that the 100 and 47 kDa proteins that interact with this subfragment are involved in the control of transcription. One possible mechanism of this transcription regulation is illustrated in Figure 6. This mechanism involves interaction between distal DNA-bound regulatory factors and the 100 kDa protein species (bound to the DNA spanning nt −558 to −514). This kind of interaction would presumably involve direct looping of DNA [Rippe et al., 1996]. This association would prevent the distal repressor elements and their interacting proteins from contact with the transcription initiation complex to inhibit transcription. The presence of the 47 kDa protein in non-collagen X-expressing cells would mask the interaction site on the 100 kDa protein, resulting in the inability of the upstream repressor complex to make contact with the 100 kDa protein. As a result, the upstream repressor complex would be free to interact with the transcription initiation complex, thus inhibiting transcription. Based on the proposed mechanism, the absence of the 44 bp fragment in reporter constructs in hypertrophic chondrocytes results in inhibition of transcription of the reporter gene because the 100 kDa protein does not have a DNA site with which to anchor itself. This model presumes that the distal repressor "complex" has a greater affinity for the 100 kDa protein than it does for the transcription initiator complex. The model also does not take into account the possibility of other enhancers in either the proximal or distal part of the promoter, which may play a supporting role in the "de-repressor" activity. The strong promoter activity conferred by the 640 bp fragment in all cell types [LuValle et al., 1993] implies that enhancer(s) exist in this fragment. We have also recently identified an enhancer element in the distal regulatory region of the chick collagen X gene (Leask and LuValle, unpublished data). An enhancer in a similar location within the human distal promoter has also recently been described [Beier et al., 1997], suggesting that some conservation of regulatory control mechanisms exists between the two species. In addition, some of the proteins interacting with upstream DNA may not be repressors, but may instead be involved in the mediation of interaction with the proteins that interact with the 44 bp subfragment. The data presented here suggest that a complex and novel control mechanism is necessary for hypertrophic chondrocyte-specific expression of the chicken collagen X gene. Future experiments will involve identifying and characterizing the regulatory proteins involved, functional analyses via ectopic expression of these proteins in developing chick embryos to define their role in vivo, and determination of a possible role for these regulatory proteins at additional control points involved in endochondral ossification.

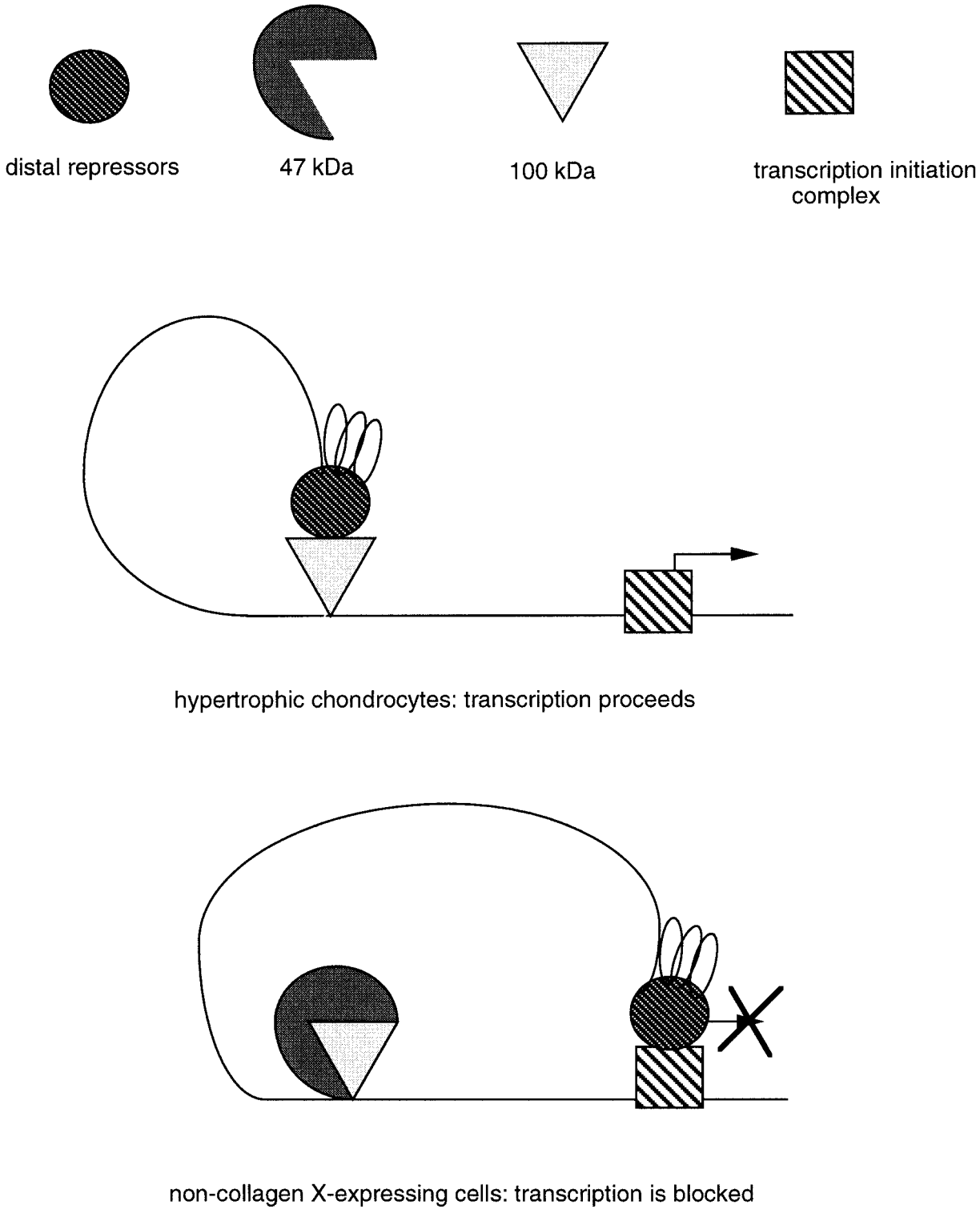


Fig. 6. Model for the role of 100 and 47 kDa proteins in control of collagen X gene transcription. The distal repressors (shown collectively as a circle) interact with the 100 kDa protein (triangle) in hypertrophic chondrocytes, which effectively inhibits the repressors from acting to turn off transcription by interacting with the transcription initiation complex (square). The distal repressors are able to inhibit transcription in non-collagen X expressing cells because their binding site on the 100 kDa protein is blocked by the presence of the 47 kDa protein (circle with large portion missing). See Discussion for details.

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