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Regulation of Human *COL9A1* Gene Expression

ACTIVATION OF THE PROXIMAL PROMOTER REGION BY SOX9*

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The *COL9A1* gene contains two promoter regions, one driving expression of a long α1(IX) chain in cartilage (upstream) and one driving expression of a shorter chain in the cornea and vitreous (downstream). To determine how the chondrocyte-specific expression of the *COL9A1* gene is regulated, we have begun to characterize the upstream chondrocyte-specific promoter region of the human *COL9A1* gene. Transient-transfection analyses performed in rat chondrosarcoma (RCS) cells, human chondrosarcoma (HTB) cells, and NIH/3T3 cells showed that the *COL9A1* promoter was active in RCS cells but not HTB or NIH/3T3 cells. Inclusion of the first intron had no effect on promoter activity. In transient-transfection analyses with promoter deletion constructs, it was found that full promoter activity in RCS cells depended on the region from −560 bp to +130 bp relative to the transcriptional start site (+1). Sequence analysis of the region from −890 bp to the transcriptional start predicted five putative SOX/Sry-binding sites. Mutation analysis revealed that two of three putative SOX/Sry binding sites within the −560 to +130 bp region are responsible for most of the *COL9A1* promoter activity in RCS cells. Co-transfection experiments with a SOX9 expression plasmid revealed that a construct containing the five putative SOX/Sry-binding sites was transactivated 20- to 30-fold in both HTB and NIH/3T3 cells. Further co-transfection experiments showed that two of the SOX/Sry-binding sites located within the −560 to +130 bp region were required for full transactivation. However, mutation and deletion analyses indicated that a region from −560 to −357 bp, which does not contain any other conspicuous SOX9 sites, is also important for full promoter activity. DNA-protein binding assays and super-shift analysis revealed that SOX9 can form a specific complex with one of the SOX/Sry-binding sites with in the −560 to +130 region.

The chondrocyte is responsible for the precise production of several different types of cartilage tissues in the developing vertebrate; including growth plate cartilage, articular cartilage, and the cartilage of the ear and trachea. In each of these situations, the elaboration of a complex and extensive extracellular matrix, which is the main functional component of cartilaginous tissues, is crucial. The expression of extracellular matrix molecules by chondrocytes must, therefore, be tightly and coordinately controlled at the level of both synthesis and degradation to ensure that the matrix is properly constructed and maintained. Part of this control occurs at the level of the regulation of chondrocyte-specific gene expression. The best studied gene in this regard is the *COL2A1* gene, which gives rise to the main fibrillar collagen in the cartilage matrix. The expression of the *COL2A1* gene is controlled through transcription factors that interact with both the promoter and the chondrocyte-specific enhancer located within the first intron (1–4). Recent work has shown that both positive and negative factors interact with the *COL2A1* gene to regulate its expression (2–8). Positive regulation of the *COL2A1* gene during chondrocyte differentiation is afforded by the interaction of members of the *S*ry-type HMG b*ox* (SOX)[1] family of transcription factors with a specific region of the intronic enhancer (5–8). Three SOX factors, L-SOX5, SOX6, and SOX9, have been shown to cooperatively activate the expression of the *COL2A1* gene, and SOX9 has been shown to be essential for normal skeletogenesis (6, 9, 10).

Despite the wealth of information concerning the control of expression of the *COL2A1* gene, relatively little is known about the transcriptional regulation of other chondrocyte-specific collagen genes, including those encoding collagens that interact with type II collagen, such as types IX and XI (11, 12). Collagen IX is a member of a subfamily of collagens termed fibril-associated collagens with interrupted-triple helices (FACITs) that also include collagens XII, XIV, XVI, and XIX (13, 14). Collagen IX is a heterotrimeric molecule composed of three polypeptide chains (α1(IX), α2(IX), and α3(IX)) each containing three collagenous domains (COL1–3) interrupted by four non-triple-helical domains (NC1–NC4) (15, 16). The NC1, NC2, and NC3 domains are of similar size in all three chains, whereas the NC4 domain of the α1(IX) chain is much larger than in the α2(IX) and α3(IX) chains (17, 18). Indeed, the NC4 domain of the α1(IX) chain is encoded by exons 1–8 in the *COL9A1* gene, which are expressed through the use of the cartilage-specific promoter region studied in the present work (19, 20). A second promoter, located between exons 6 and 7, is utilized in other tissues to express a short form of the α1(IX) chain that lacks the large NC4 domain (19, 20). Interestingly, mice that lack type IX collagen develop normally but exhibit a late-onset form of joint degeneration similar to osteoarthritis, suggesting a stabilizing role for type IX collagen in cartilage (21, 22).

The purpose of this study was to examine the regulatory elements located within the proximal promoter region of the

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[1] The abbreviations used are: SOX, *S*ry-type HMG b*ox*; EMSA, electrophoretic mobility-shift assay; RCS, rat chondrosarcoma; HTB, human chondrosarcoma.

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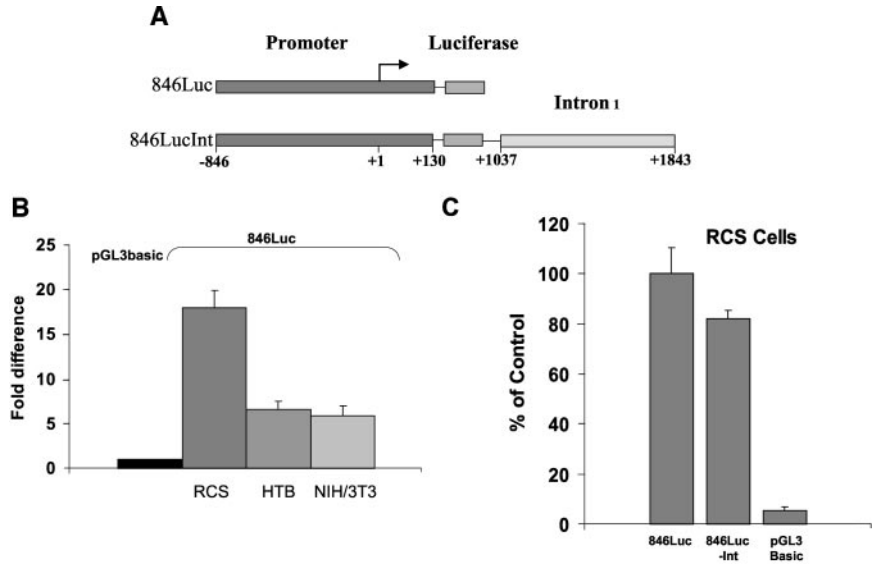

FIG. 1. **Relative activity of the human *COL9A1* promoter in RCS, HTB, and NIH/3T3 cells.** *A*, schematic illustration of the human *COL9A1* promoter and promoter-intron luciferase-reporter constructs. The 846Luc construct contains an 846-bp *COL9A1* gene fragment encompassing the promoter region and part of the first exon (not including the ATG translational start codon) and the 846LucInt construct contains an 807-bp gene fragment encompassing the first intron (not including the 5′- and 3′-splice site sequences). *B*, activity of the 846Luc construct in RCS, HTB, and NIH/3T3 cells. The cells were co-transfected with 10 μg of the 846Luc construct or the pGL3basic empty vector and 2 μg of the pCMVβ plasmid. *C*, effect of the first intron on promoter activity in RCS cells. The 846Luc or 846LucInt constructs, or the pGL3basic empty vector were co-transfected with the pCMVβ plasmid into RCS cells. Transfected cells were incubated for 48 h and then luciferase and β-galactosidase activities were determined as described under "Materials and Methods." Data are presented as average -fold difference of luciferase activity *versus* control (pGL3basic) vector ± S.D., or as average percent of control (pGL3basic) ± S.D.

human *COL9A1* gene in chondrocytic cells. We found that a 976-bp promoter fragment from the human *COL9A1* gene was able to drive expression of reporter genes in RCS cells. We also found that this promoter region can be transactivated to high levels by SOX9 in nonchondrocytic NIH/3T3 and human chondrosarcoma cells (HTB). We show that the SOX9 transactivation depends on two of five putative SOX/Sry-binding sites by mutational analysis and that a third region that does not contain any obvious SOX/Sry-binding sites is also important for full promoter activity. Finally, we provide evidence that SOX9 can interact with one of the functionally active aforementioned SOX/Sry-binding sites.

MATERIALS AND METHODS

Cell Culture—Rat chondrosarcoma cells (RCS) were a kind gift from Dr. Benoit de Crombrugghe (23). HTB human chondrosarcoma cells (SW1353) and NIH/3T3 fibroblasts were obtained from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose), 10% fetal bovine serum supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and ascorbic acid (50 μg/ml). Cells were split at 70–80% confluence, and the medium was replaced every 3–4 days.

COL9A1-Luciferase Constructs—The human *COL9A1* proximal promoter region and first intron were obtained by PCR using a P1 phage clone (P1-A) that contains the 5′ region of the human *COL9A1* gene (a kind gift from Dr. Lena Ala-Kokko; ref. 20) and the following primers. For the promoter region: primer DS37, 5′-GATCGAATTCAGTAGGGGGCTTGATGTTA-3′ (forward) and primer DS38, 5′-GATCCTCGAGTTCCCAGTTGATTTTCTTTG-3′ (reverse), and for the first intron: primer DS39, 5′-GATCGTCGACAAGACAATAACCCTGGAAAGA-3′ (forward) and primer DS40, 5′-GATCAAGCTTTGAAACAGGAGTCCCCGCAGA-3′ (reverse). The primers for the promoter region contained an *Eco*RI and a *Xho*I site, respectively, and the primers for the first intron contained a *Sal*I and a *Hin*dIII site, respectively, to facilitate cloning. The PCR-generated promoter fragment (976 bp) contained the region from −846 to +130 relative to the transcriptional start site, and the first intron fragment (807 bp) contained from position +1037 to +1843 in the human *COL9A1* gene sequence (excluding the splice-site sequences) (20). The promoter fragment was cloned into the *Nhe*I-*Xho*I sites of the pGL3basic luciferase-reporter vector (Promega, Madison, WI) to generate the full-length 846Luc promoter construct. To generate the 846LucInt construct, the intron fragment was cloned into the *Hin*dIII-*Sal*I sites of 846Luc. The various *COL9A1* promoter-deletion constructs were generated by PCR using different forward primers (see below) in combination with the same reverse primer (DS38, see above). For construct 588Luc, 5′-AATGATTGTTGGGTGTTAGAC-3′; for construct 560Luc, 5′-GATCGCTAGCCTTTGATACCTCATTT-3′; for construct 357Luc, 5′-GTGGGCACATTTTTACTGGA-3′; for construct 167Luc, 5′-TTCCCCTGTAAATCCCTCCTTC-3′, and for construct 107Luc, 5′-GATCGCTAGCCTGGGCTCAGAGCGCT-3′. In the case of the primers for constructs 560Luc and 107Luc, *Nhe*I sites were placed into the 5′ end to facilitate cloning. The deletion-PCR products for 588Luc, 357Luc, and 167Luc were cloned into the *Sma*I-*Xho*I sites of pGL3basic, and the PCR products for 560Luc and 107Luc were cloned into the *Nhe*I-*Xho*I sites of pGL3basic. For the pGL3ProC construct, an 85-bp PCR product encompassing the region from −559 to −475 was cloned into the pGL3promoter vector (Promega), which contains the SV40 promoter linked to the luciferase reporter gene. The pGL3ProD construct was made in a similar manner by inserting a 96-bp PCR fragment encompassing the region from −356 to −261 into the pGL3promoter vector. All PCR products were verified by sequencing on an ABI automatic DNA sequencer (PerkinElmer Life Sciences).

Transient Transfection and Luciferase Assays—Seven to ten μg of the various *COL9A1* reporter constructs, 2 μg of the SOX expression vectors (a kind gift from Dr. Veronique Lefebvre, 6), and 2 μg of the pCMVβ reporter vector (Clontech), as a control for transfection efficiency, were co-transfected into either RCS, HTB, or NIH/3T3 cells by the calcium phosphate precipitation method using the Profection kit (Promega). The cells were plated 24 h before transfection at a density of 5×10^5 cells per 10 cm^2 dish. The DNA-$CaPO_4$ precipitate was left on the cells for 16–18 h, after which the cells were washed 3 times with phosphate-buffered saline followed by the addition of fresh media. Forty-eight h after transfection, the cells were harvested and luciferase assays were performed with a luciferase assay kit (Promega) and a Turner Designs TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). β-Galactosidase assays were performed spectrophotometrically with a β-galactosidase enzyme assay system (Promega). Protein concentrations in the cell lysates were determined using the Coomassie Blue protein assay (Pierce). Luciferase activity was normalized to both β-galactosidase activity and protein concentration. Transfection data represent at least two independent experiments each performed in triplicate, except where indicated.

Nuclear Extracts and Electrophoretic Mobility-shift Assays—Nuclear extracts were prepared according to the method of Dignam *et al.* (24) using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Briefly,

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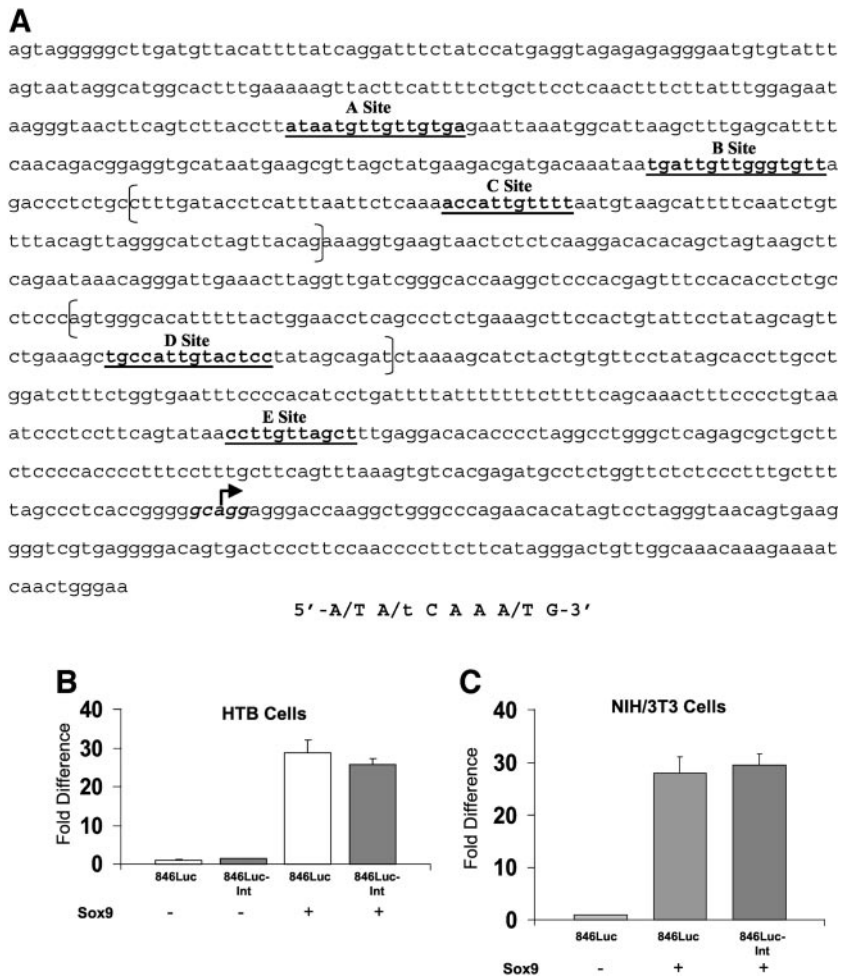

FIG. 2. **SOX9 transactivates the human *COL9A1* proximal promoter region in NIH/3T3 and HTB cells.** *A*, sequence of the human *COL9A1*proximal promoter region. Shown in *bold* and *underlined* are the 5 putative SOX/Sry-binding sites designated A–E. *Brackets* denote the regions that were used to construct the pGL3proC and pGL3proD constructs in Fig. 6. Shown below the sequence for comparison is the HMG-SOX/Sry-binding site consensus sequence (5). *B*, transactivation of the 846Luc and 846LucInt reporter constructs in HTB cells by co-transfection with a SOX9 expression vector. Ten micrograms of either 846Luc or 846LucInt were transfected into HTB cells with 2 μg of pBluescript or a SOX9 expression vector and 2 μg of pCMVβ. *C*, transactivation of 846Luc and 846LucInt by co-transfection with a SOX9 expression vector in NIH/3T3 cells. NIH/3T3 cells were transfected as in *B*. Transfected cells were incubated for 48 h, and luciferase and β-galactosidase activities were determined as described in "Materials and Methods." Data are presented as average -fold difference of luciferase activity *versus* control (846Luc, no SOX9) ± S.D.

cells were placed in hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol) and incubated on ice for 15 min. Igeapal CA-630 was added to a final concentration of 0.6%, and the mixture was vortexed vigorously for 10 s. Nuclei were recovered by centrifugation at 3,300 × *g* for 30 s at 4 °C and extracted in buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM $MgC1_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol for 30 min at 4 °C by gentle shaking. The extract was then centrifuged for 15 min at 25,000 × *g*, and the supernatant was frozen at −70 °C. All buffers contained a protease inhibitor mixture (2 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 1.4 pM trans-epoxysuccinyl-L-leucylamido [4-guanidinobutane], 130 pM bestatin, 1 μM leupeptin, and 0.3 pM aprotinin; Sigma-Aldrich).

Electrophoretic mobility-shift assays (EMSAs) were carried out as previously described with minor modifications (25). Briefly, binding reactions consisted of 12.5 mM Hepes, pH 7.9, 50–100 mM NaCl, 5% glycerol, 2 mg/ml bovine serum albumin, 2 μg poly(dG-dC), 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 ng of ^{32}P-end labeled double-stranded oligonucleotide probe, and 10–15 μg of nuclear protein. Binding reactions were incubated for 30 min at 21 °C and then loaded onto 4% acrylamide-0.25× Tris borate-EDTA gels and electrophoresed at 200 V for 2 h. For competition analyses, 50-fold excess of cold-competitor probe was included in the binding reaction. For super-shift analyses, 3 μl of a rabbit polyclonal anti-SOX9 antibody (a kind gift from Dr. Veronique Lefebvre; Ref. 6) was included in the binding reaction.

RESULTS

Human COL9A1 Promoter Activity in Different Cells and the Effect of the First Intron on COL9A1 Promoter Activity—A luciferase reporter gene construct (846Luc) containing a 976-bp *COL9A1* gene fragment that includes 846 bp of the promoter and 130 bp of the first exon (up to but not including the ATG initiation codon) (Fig. 1*A*) was transfected into RCS, HTB chondrosarcoma, and NIH/3T3 cells to determine the relative promoter activity as compared with the pGL3basic vector (no promoter). The 846Luc construct exhibited a high level of promoter activity in the chondrocytic RCS cells (~18-fold over the vector alone, Fig. 1*B*), however, in HTB and NIH/3T3 cells, the *COL9A1* promoter activity was substantially lower (Fig. 1*B*). To examine whether the first intron of the human *COL9A1* gene contains transcriptional regulatory elements, an 807-bp fragment containing the first intron (excluding the splice-site sequences at the 5′ and 3′ ends) was inserted into the 846Luc construct to obtain 846LucInt. The two constructs, 846Luc and 846LucInt, were then separately transfected into RCS cells. As can be seen in Fig. 1*C*, inclusion of the *COL9A1* first intron fragment did not result in any significant difference in promoter activity.

Transactivation of the COL9A1 Promoter by SOX9—SOX transcription factors have been shown to be important for the regulation of chondrocyte-specific gene expression. Sequence analysis of the proximal promoter region of the human *COL9A1* gene with the MatInspector program (26) revealed five putative SOX/Sry-binding sites (Fig. 2*A*), whereas analysis of the first intron did not reveal any recognizable SOX/Sry-binding sites. To determine whether SOX9 regulates the activity of the *COL9A1* gene, either the 846Luc or the 846LucInt construct were co-transfected with a SOX9 expression construct into HTB and NIH/3T3 cells. SOX9 overexpression in these cells activated the 846Luc and 846LucInt constructs by ~25- to 30-fold (Fig. 2, *B* and *C*). However, there was no difference in activation between the two constructs in response to SOX9, indicating that the observed transcriptional activation depends on the proximal-promoter region and that the first

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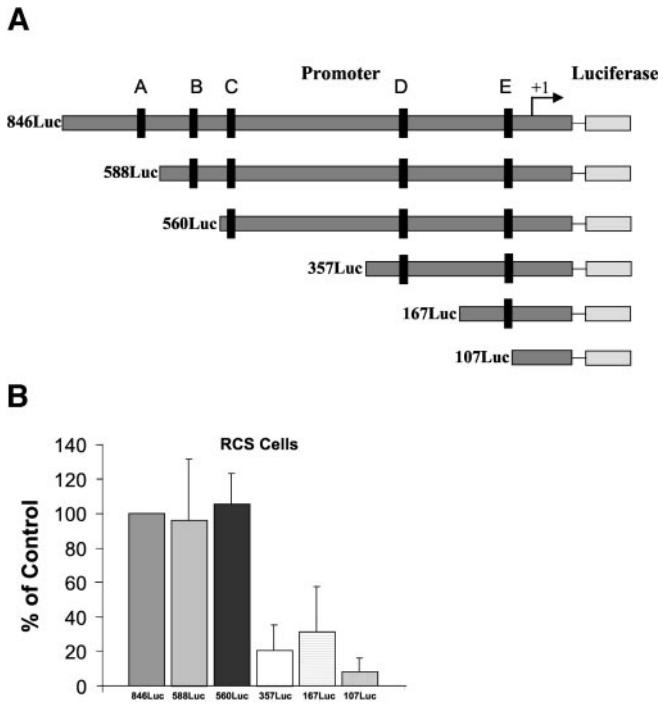

FIG. 3. **Deletion analysis of the human *COL9A1* proximal promoter region in RCS cells.** *A*, schematic illustration of the various 5′-deletion constructs employed in the analyses. The deletion constructs were generated as described under "Materials and Methods." *B*, relative luciferase activity of the *COL9A1* promoter-deletion constructs in RCS cells. RCS cells were transfected with 10 μg of each deletion construct and 2 μg of the pCMVβ plasmid. Transfected cells were incubated for 48 h, and luciferase and β-galactosidase activities were determined as described under "Materials and Methods." Data are presented as average percent of control (846Luc) ± S.D. Data represent 3–4 experiments performed in triplicate.

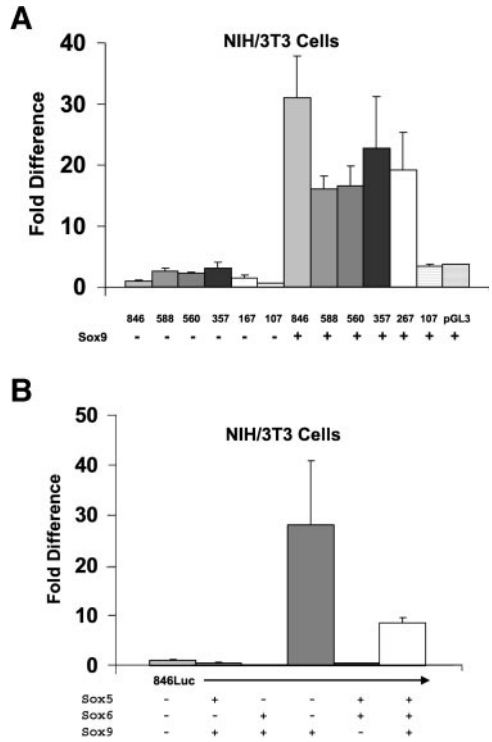

FIG. 4. **Transactivation of *COL9A1*-promoter-deletion constructs by SOX9 in NIH3T3 cells.** *A*, the various *COL9A1* promoter-deletion constructs (Fig. 3*A*) were co-transfected into NIH/3T3 cells with 2 μg of pBluescript vector or with a SOX9 expression vector and 2 μg of the pCMVβ plasmid. *B*, transactivation of the 846Luc construct in NIH/3T3 cells by L-SOX5 and SOX6. NIH/3T3 cells were co-transfected with 7 μg of the 846Luc construct and either 2 μg of pBluescript or expression vectors for L-SOX5, SOX6, or SOX9 and 2 μg of the pCMVβ plasmid. Transfected cells were incubated for 48 h, and luciferase and β-galactosidase activities were determined as described under "Materials and Methods." Data are presented as average -fold difference of luciferase activity *versus* control (846Luc alone) vector ± S.D.

intron did not have any influence on the transcriptional activity of the gene under these conditions.

Deletion Analysis of the COL9A1 Proximal Promoter Region—To delineate which regions of the *COL9A1* promoter region are responsible for promoter activity and SOX9 transactivation, a series of *COL9A1*-promoter-luciferase 5′-deletion constructs were employed (Fig. 3*A*). The deletion constructs were generated such that the five putative SOX/Sry-binding sites, designated A–E, were sequentially removed (Figs. 2*A* and 3*A*). To determine the effect of the deletions on promoter activity, the full-length construct (846Luc) and the five deletion constructs (588Luc, 560Luc, 357Luc, 167Luc, and 107Luc) were transfected into RCS cells (Fig. 3*B*). Deletion of up to 286 bp from the 5′ end, including the two distal putative SOX/Sry-binding sites (588Luc and 560Luc, sites *A* and *B* in Fig. 3*A*) did not result in any substantial changes in activity in RCS cells (Fig. 3*B*). However, when the region containing the three distal putative SOX/Sry sites was removed (357Luc, sites *A*–*C* in Fig. 3*A*), the promoter activity was reduced to 20% of control (Fig. 3*B*). Deletion of the region encompassing the four distal sites (167Luc, sites *A*–*D* in Fig. 3*A*) resulted in slightly increased expression as compared with the 357Luc construct (sites A–C deleted) and an overall expression level of 31% of control (Fig. 3*B*). Finally, deletion of the region encompassing all five sites (107Luc in Fig. 3*A*) resulted in a 5.5-fold decrease in promoter activity (9% of control, Fig. 3*B*). We conclude that the region encompassing the SOX/Sry-binding sites C–E is important for full promoter activity in RCS cells.

To determine which of the putative SOX/Sry sites within the proximal promoter region were responsible for the observed transactivation by SOX9, the various deletion constructs were co-transfected into NIH/3T3 cells along with a SOX9 expression vector. Fig. 4*A* shows the results of this experiment. All of the deletion constructs except for the 107Luc construct, which does not contain any SOX/Sry-binding sites, were transactivated by SOX9 overexpression. The full-length 846Luc construct displayed the strongest response to SOX9 co-transfection, exhibiting a ~30-fold increase in activity, whereas the deletion constructs that contained at least one putative SOX/Sry-binding site (588Luc, 560Luc, 357Luc, and 167Luc) displayed a 15- to 20-fold increase in activity over control.

Two other SOX proteins, L-SOX5 and SOX6 have been shown to cooperate with SOX9 in the transcriptional activation of the *COL2A1* gene through the intronic enhancer region (6). To determine whether L-SOX5 and SOX6 play a role in the activation of the *COL9A1* proximal promoter region, the 846Luc construct was co-transfected into NIH/3T3 cells with L-SOX5, SOX6, and SOX9 expression constructs. Fig. 4*B* demonstrates that neither L-SOX5 or SOX6 alone or in combination activate the *COL9A1* proximal promoter. Interestingly, when L-SOX5, SOX6, and SOX9 were co-transfected together with the 846Luc construct, transactivation of the promoter was attenuated as compared with co-transfection with SOX9 alone (Fig. 4*B*).

Mutations in the SOX/Sry-binding Sites Suppress Transcriptional Activation—To define more precisely the role of the putative SOX/Sry-binding sites within the *COL9A1* proximal promoter region, point mutations were introduced into sites C, D, and E (Fig. 5*A*). In each case, a 4-bp change was introduced into the core SOX/Sry-binding sites employing PCR-based mutagenesis. The intact (wild-type) promoter construct (846Luc)

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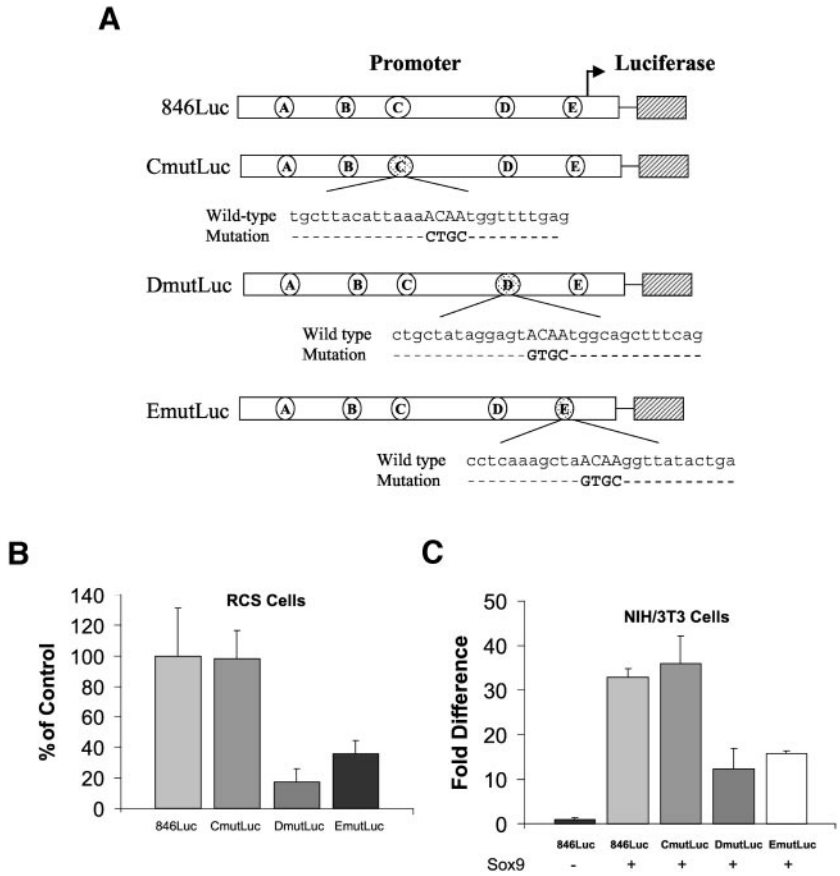

FIG. 5. **Mutations in the SOX/Sry-binding sites D and E reduce transcriptional activity and transactivation of the *COL9A1* promoter by SOX9 in RCS and NIH/3T3 cells.** *A*, schematic illustration of the point mutations introduced into the SOX/Sry binding sites C, D, and E, to generate constructs CmutLuc, DmutLuc, and EmutLuc. *B*, mutations in sites D and E reduce transcriptional activity of the *COL9A1* promoter in RCS cells. The wild-type 846Luc construct or the CmutLuc, DmutLuc, and EmutLuc constructs (10 μg) were transfected into RCS cells along with 2 μg of the pCMVβ plasmid. *C*, mutations in sites D and E reduce the transactivation by SOX9 in NIH/3T3 cells. The wild-type 846Luc construct or the CmutLuc, DmutLuc, and EmutLuc constructs (10 μg) were co-transfected into NIH/3T3 cells along with 2 μg of pBluescript or a SOX9 expression vector and 2 μg of the pCMVβ plasmid. Transfected cells were incubated for 48 h, and luciferase and β-galactosidase activities were determined as described under "Materials and Methods." Data are presented as average percent luciferase activity of control (846Luc) ± S.D. or as average -fold difference of luciferase activity *versus* control (846Luc, no SOX9) ± S.D.

and each of the mutant constructs (CmutLuc, DmutLuc, and EmutLuc) were transfected separately into RCS cells to assess the effect of the mutations on promoter activity. Fig. 5*B* demonstrates that mutations in site C had no effect on *COL9A1* promoter activity. In contrast, mutations in sites D and E reduced the promoter activity to 17 and 36% of control, respectively. Similarly, in co-transfection experiments with a SOX9 expression vector, the wild-type 846Luc and CmutLuc constructs were transactivated ~30- to 35-fold over control (Fig. 5*C*), whereas the DmutLuc and EmutLuc constructs were activated to only 1/3 of those levels (10- to 12-fold, Fig. 5*C*) by SOX9 co-transfection. These results demonstrate that the SOX/Sry-binding sites D and E are necessary for full promoter activity in RCS cells and for full transactivation by SOX9 in NIH/3T3 cells.

Although the results from the deletion analysis indicated that the region encompassing site C was important for full activity (construct 357Luc, Fig. 3*B*), the mutational studies were contradictory because they showed that site C itself was dispensable for promoter activity (CmutLuc construct, Fig. 5, *B* and *C*). To clarify these results, either an 85-bp fragment encompassing site C or a 96-bp fragment encompassing site D (see *brackets* in Fig. 2*A*) were inserted into the pGL3promoter vector, which contains the SV40 promoter (pGL3proC and pGL3proD, Fig. 6*A*). When these constructs were transfected into RCS cells, the pGL3proC construct displayed essentially the same activity as the pGL3promoter vector alone, whereas the pGL3proD construct was ~16-fold more active (Fig. 6*B*). We conclude that along with SOX/Sry binding sites D and E, there must be another positive-acting element within the promoter region from −560 to −357 relative to the transcriptional start site that is necessary for full promoter activity in RCS cells.

SOX9 Specifically Binds to SOX/Sry-binding Site D—To determine whether SOX9 interacts with the putative SOX/Sry-

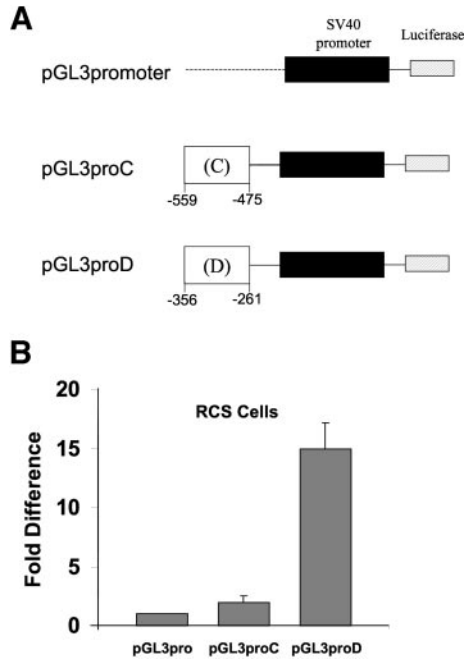

FIG. 6. **SOX/Sry-binding site-D can activate a heterologous promoter in RCS cells.** *A*, schematic illustration of the pGL3promoter constructs in which fragments encompassing sites C or D were placed in front of the SV40 promoter (see *bracketed* regions in Fig. 2*A*). *B*, transfection of RCS cells with the pGL3promoter, pGL3proC, or the pGL3proD constructs. Ten micrograms of either the pGL3promoter, pGL3proC, or the pGL3proD construct along with 2 μg of the pCMVβ plasmid were transfected into RCS cells. Transfected cells were incubated for 48 h, and luciferase and β-galactosidase activities were determined as described under "Materials and Methods." Data are presented as average -fold difference of luciferase activity *versus* control (pGL3promoter) ± S.D.

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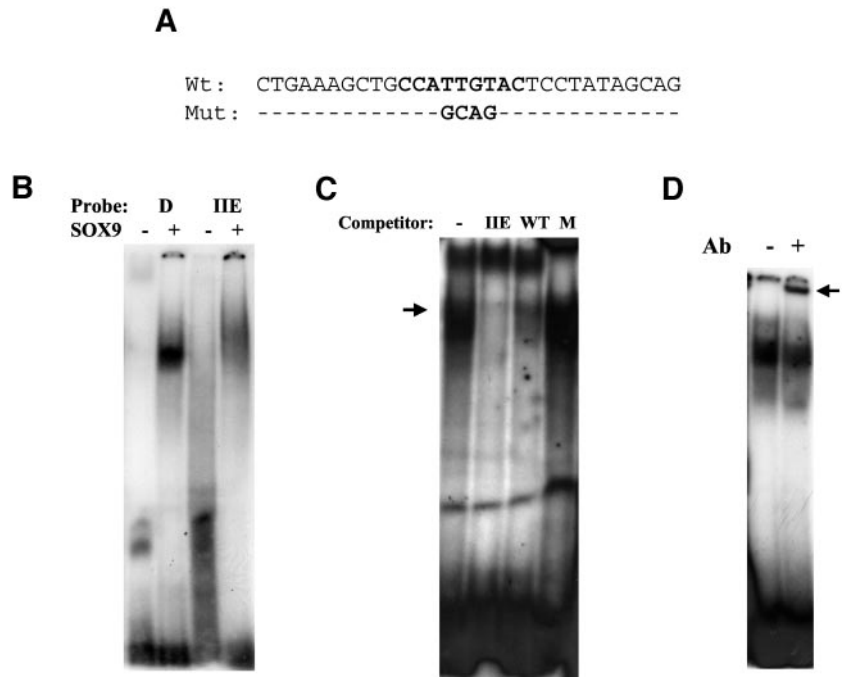

FIG. 7. **SOX9 specifically interacts with the SOX/Sry-binding site-D in the human *COL9A1* promoter.** *A*, sequence of the wild-type and mutated SOX/Sry-binding site-D probes employed in the EMSA experiments. Nucleotides in **bold** indicate the SOX/Sry-binding site. *B*, a specific DNA-protein complex forms with a probe encompassing the SOX/Sry-binding site D in NIH/3T3 cells transfected with a SOX9 expression construct. Nuclear extracts were prepared from NIH/3T3 cells that had either been mock-transfected or transfected with a SOX9 expression vector. The nuclear extracts were subjected to EMSA with either a labeled SOX/Sry-binding site-D probe (*D*) or a probe containing the SOX/Sry-binding sites from the human *COL2A1* gene enhancer (*IIE*). *C*, DNA-binding competition analysis of the SOX/Sry-binding site-D. EMSA was performed with RCS nuclear extract and the addition of 50-fold excess unlabeled human *COL2A1* enhancer probe (*IIE*), wild-type SOX/Sry-binding site-D probe (*WT*), or mutated SOX/Sry-binding site-D probe (*M*). *Arrow* indicates specific DNA-protein complex that is competed with excess IIE and WT probes but not with excess M probe. *D*, antibody supershift analysis of the SOX-Sry-binding site-D. Nuclear extracts from NIH/3T3 cells transfected with a SOX9 expression construct were subjected to a antibody supershift assay with anti-SOX9 antibodies. *Arrow* indicates super-shifted SOX9-D-site complex. Nuclear extracts were prepared and EMSAs were performed as described under "Materials and Methods."

binding site-D, nuclear extracts were prepared from NIH/3T3 cells that had been transfected with SOX9 expression constructs, and electrophoretic mobility shift assays were performed. Fig. 7*A* shows the sequence of the SOX/Sry-binding site D probe used in these experiments. Fig. 7*B* shows that specific binding to probes containing SOX/Sry-binding site D or to a probe containing the SOX binding sites from the *COL2A1* gene enhancer region were detected only in NIH/3T3 cells that had been transfected with a SOX9 expression construct and not in control cells. Competition analysis revealed that a specific DNA-protein complex forms with SOX/Sry-binding site-D in nuclear extracts from RCS cells, and this complex can be competed for with excess wild-type SOX/Sry-binding site-D probe and excess probe containing the SOX-binding sites from the human *COL2A1* gene but not with a mutant SOX/Sry-binding site-D probe (Fig. 7*C*). Finally, the DNA-protein complex that forms with the wild-type SOX/Sry-binding site-D probe and nuclear extract from NIH/3T3 cells transfected with a SOX9 expression construct can be supershifted with anti-SOX9 antibodies (Fig. 7*D*).

DISCUSSION

We demonstrate here that the proximal-promoter region of the human *COL9A1* gene can drive expression of a reporter gene in chondrocytic RCS cells but not in the nonchondrocytic HTB and NIH/3T3 cell lines. We also showed that the *COL9A1* proximal-promoter region can be transactivated by SOX9 and that the transactivation depends, in part, on two SOX/Sry-binding sites (sites D and E). Also, full promoter activity depends on a region located between −560 to −357 relative to the transcriptional start site. Further work revealed that SOX9 can specifically bind to at least one of the two sites (SOX/Sry-binding site-D) as demonstrated by EMSA competition and supershift analyses. Our results strengthen the notion that SOX9 is a master regulatory factor for chondrocyte-specific gene expression.

An interesting difference between the regulation of the *COL9A1 versus* the *COL2A1* promoter was observed when the L-SOX5 and SOX6 expression constructs were included in the co-transfection experiments. L-SOX5 and SOX6 did not stimulate the transcriptional activity of our *COL9A1* promoter construct either alone or in combination. Even more intriguing was the finding that when the L-SOX5 and SOX6 vectors were co-transfected along with the SOX9 expression vector, the activity of the *COL9A1* promoter was attenuated. In contrast to this, L-SOX5 and SOX6 have been shown to stimulate the transcriptional activity of the *COL2A1* enhancer either alone or in combination (6). Furthermore, when L-SOX5 and SOX6 are expressed along with SOX9 in co-transfection assays, the activity of the *COL2A1* gene was stimulated in an enhancer-dependent fashion to higher levels than that observed with the individual SOX factors (6). It is interesting to speculate that this might be a mechanism by which the chondrocyte could achieve different levels of *COL9A1* and *COL2A1* gene expression utilizing the same transcription factors. Indeed, a similar type of regulation has been observed with respect to the *COL2A1* gene. The transcription factor cKrox was shown to interact with multiple sites within the enhancer region, resulting in up-regulation of the gene in differentiated chondrocytes, whereas cKrox-binding sites located in the promoter were responsible for down-regulation of the gene in de-differentiated chondrocytes (2). However, the *COL2A1* gene is also regulated by other negatively acting transcription factors. The transcrip-

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tion factor δEF1 has been shown to repress *COL2A1* gene expression through sites in the promoter region in limb-bud mesenchymal cells before differentiation occurs (3). The zinc-finger transcription factor αA-crystallin binding protein-1 is also a negative regulator of the *COL2A1* gene, acting through sites located in the enhancer region (4). Certainly, more work is needed to elucidate the complex network of control mechanisms involved in chondrocyte-specific gene expression.

The structure of the promoter for *COL9A1* is reminiscent of that of the *COL11A2* gene. A cluster of five SOX/Sry-binding sites were also characterized in the proximal promoter region of the *COL11A2* gene, located between −742 bp and the transcriptional start site (27). These sites were found to bind a specific protein complex in RCS nuclear extracts that contained SOX9 and also were able to direct reporter gene expression to cartilaginous tissues in transgenic mice (27). Other work has implicated a 60-bp segment within the first intron that is important for *COL11A2* gene expression. This segment was also shown to interact with SOX9 and promote cartilage-specific expression of reporter genes in transgenic mice (28). Interestingly, the SOX/Sry sites located in the *COL11A2* proximal promoter region differ from the sites studied in the present work in that they are much closer to one another. Furthermore, we found that the first intron of the *COL9A1* gene did not augment the activity of the promoter region. However, our experiments do not rule out a role for the first intron in regulating *COL9A1* gene expression *in vivo* because we have not investigated the activity of the *COL9A1*-reporter constructs in the context of transgenic mice.

As mentioned above, the arrangement of the SOX/Sry-binding sites within the *COL9A1* proximal-promoter region differ from those found in the *COL11A2* promoter and also from the SOX/Sry-binding sites located in the *COL2A1* intronic enhancer (5–8, 27–30). In the case of the *COL11A2* gene, the five SOX/Sry-binding sites are found within a region of ~130 bp (27). However, the distal three sites are within 45 bp, and the proximal two sites are separated by only 3 bp. Similarly, the four SOX/Sry-binding sites within the *COL2A1* intronic enhancer are spaced over a 48-bp region (5–8). In contrast to this, the five SOX/Sry-binding sites within the *COL9A1* promoter span a region of 565 bp. The transient-transfection studies presented here rule out sites A and B as being important for full promoter activity; however, the remaining three SOX/Sry-binding sites are scattered over a 415-bp stretch, still substantially less compact than the *COL11A2* and *COL2A1* genes. Whether this arrangement has any bearing on the relative levels of expression of these genes or the developmental timing of their expression remains to be determined, as does the placement of the SOX/Sry-binding sites within the proximal-promoter region *versus* an enhancer region or both.

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Regulation of Human *COL9A1* Gene Expression: ACTIVATION OF THE PROXIMAL PROMOTER REGION BY SOX9

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