Transcriptional activation of cartilage oligomeric matrix protein by Sox9, Sox5, and Sox6 transcription factors and CBP/p300 coactivators

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1. ABSTRACT

The gene for cartilage oligomeric matrix protein (COMP) encodes a noncollagenous matrix protein that is expressed predominantly in cartilage. COMP gene expression is deficient in the Sox9-null mouse, but the molecular mechanism remains unknown. We have previously delineated a 30-bp negative regulatory element (NRE) and a 51-bp positive regulatory element (PRE) in the regulatory region of the COMP gene. Subsequently we isolated LRF transcription repressor as an NRE-binding protein and established that LRF inhibits COMP gene expression via recruiting histone deacetylase 1 (HDAC1) to the COMP promoter. In this study we demonstrated that Sox9, an essential transcription factor of chondrogenesis, binds to the COMP promoter at the PRE in which 13 nucleotides (TGTTTACCTTGTG) are required for the binding of Sox9. Sox9 activates COMP gene expression and this activation is PRE-dependent. Sox9 is required for COMP gene expression during chondrogenesis, since repression of Sox9 expression using the small interfering RNA approach inhibited COMP gene expression. In addition, activation of COMP gene expression by Sox9 requires the participation of transcription factors Sox5 and Sox6 as well as the coactivators CBP and p300 histone acetylase. It appears that there exists a balance between LRF repressor and Sox9 activator in the control of COMP gene, since transactivation of COMP gene by Sox9 was abolished by the coexpression of LRF, and excess Sox9 overcame the LRF-mediated inhibition. This study provides the first evidence that Sox9 directly associates with COMP gene promoter and that mediation of COMP gene activation by Sox9 involves Sox5, Sox6, CBP, and p300 coactivators.

2. INTRODUCTION

The differentiation of uncommitted mesenchymal stem cells into musculoskeletal tissues, including chondrocytes, osteoblasts, tenocytes, and ligament cells, is a fundamental molecular event of both embryonic development and repair of cartilage, ligament, tendon, and bone (19, 20). A clearer understanding of the regulation of the differentiation of musculoskeletal tissues has potential clinical applications for designing biologically based treatment for several orthopaedic conditions, including fractures, large segmental cartilage defects, arthritis, and tumors.

After commitment to the chondrocyte lineage, mesenchymal cells undergo condensation, cease expression of type I collagen, and differentiate into a chondrocytic phenotype characterized by expression of collagen types II, IX, and XI and the proteoglycan aggrecan (19, 20). There appears to be a transition cell between type I collagen (expressing mesenchymal cells) and type II collagen (expressing chondrocytes). Such cells are characterized by lack of expression of type II collagen and by abundant expression of cartilage oligomeric matrix protein (COMP) (11, 12, 14-16, 33, 52, 59). These cells may represent musculoskeletal precursor cells that have the potential to subsequently differentiate into a variety of musculoskeletal cell types. Little is known about the generation of these potential precursor cells. Examination of the transcriptional regulation of COMP may shed light on the signals that control the differentiation of uncommitted precursor cells into committed cells that can subsequently differentiate into several COMP-expressing cell types.

The gene for COMP encodes a pentameric noncollagenous matrix protein (16, 17, 33, 52) expressed predominantly in articular cartilage (16, 33, 52, 59). COMP has been implicated in the regulation of chondrogenesis in a micromass culture of mesenmchymal stem 10T1/2 cells and in limb development *in vivo* (42, 43). Mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia (6-9, 30, 31, 62), autosomal-dominant forms of short-limb dwarfism characterized by short stature, normal facies, epiphyseal abnormalities, and early-onset osteoarthrosis (reviewed in (21, 35, 53)). Accumulating evidence suggests that COMP may function to stabilize the extracellular matrix of articular cartilage by specific cation-dependent interactions with matrix components, including collagen types II and IX and fibronectin (6-9, 30, 31, 49, 57, 62). COMP demonstrates significant expression in chondrocytes, osteoblasts, tenocytes, and ligament cells, but not in undifferentiated mesenchymal cells (4, 12, 13, 15, 16, 18, 22, 32, 39, 52, 59, 70). Delineation of cis-elements in the COMP promoter necessary for expression in any of these tissues may provide DNA probes for identifying transcriptional regulators that contribute to the selection of a musculoskeletal precursor cell from uncommitted mesenchymal cells.

To begin to understand the mechanisms controlling this process, we previously cloned approximately 1.9 kilobases of the 5′ flanking region of the murine COMP promoter and identified cis-elements necessary for expression in chondrocytes. A COMP promoter fragment containing approximately 1.9 kilobases of the 5′ flanking sequence is specifically active in rat chondrosarcoma (RCS) cells. Comprehensive analyses of the regulatory region of the COMP gene led to the identification of a 30-bp negative regulatory element (NRE, -1775 to -1746) and a 51-bp positive regulatory element (PRE, -125 to -75) (40). A functional genetic screen with the 30-bp NRE as bait resulted in the isolation of leukemia/lymphoma-related factor (LRF) transcriptional repressor as a binding protein at the NRE (48). Subsequent studies revealed that LRF inhibits COMP gene expression and chondrogenesis via recruiting histone deacetylase 1 (HDAC1) to the COMP promoter. In addition to inhibiting COMP gene expression, LRF also suppresses transcription driven by promoters for the type I collagen α1, type I collagen α2, fibronectin, and elastin genes (69); Recently, LRF was shown to act as a proto-oncogene via suppressing the expression of the Alternative reading frame protein (ARF), a tumor suppressor (50, 51).

It is interesting to note that the 51-bp positive regulatory element contains three putative binding sites of Sox9, a master regulator of chondrocyte differentiation. The transcription factor Sox9 has been demonstrated to play an essential role in chondrocyte differentiation (5, 67, 68). Mouse knockout experiments have shown that in the absence of Sox9 a blockage in cartilage differentiation occurs at the mesenchymal condensation stage. Overexpression of Sox9 in chondrocytes of mouse embryos produces a similar phenotype of dwarfism with decreased chondrocyte proliferation, delayed hypertrophic chondrocyte differentiation, and endochondral bone formation (2). Sox9 is also required for the activation of chondrocyte-specifc marker genes, including collagen type II, cartilage link protein, and cartilage-derived retinoic acid–sensitive protein (CD-RAP). There is emerging evidence that Sox9-mediated gene activation requires the participation of its cofactors; for example, transcription coactivator CBP/p300, which exhibits histone acetylase activity, has been reported to associate with Sox9 and to affect the transactivation of type II collagen and CD-RAP gens by Sox9 (24, 25, 38, 64). In addition, it has been shown that Sox5 and Sox6, two downstream transcription factors of Sox9, are also required for the expression of chondrocyte-specific genes and for chondrogenesis (1, 36, 37, 46).

The aims of this study were to characterize the binding of Sox9 to COMP promoter, to determine whether Sox9 activates COMP gene expression and whether it is required for the induction of the COMP gene during chondrogenesis, and to elucidate the molecular events by which Sox9 regulates COMP gene expression.

3. MATERIAL AND METHODS

3.1. Preparation of nuclear extracts

Nuclear extracts were prepared from the Swarm rat chondrosarcoma (RCS) cell lines transfected with an

expression plasmid pFlag-Sox9. Cells were harvested by trypsinization, washed in phosphate-buffered saline (PBS), pelleted, and resuspended in lysis buffer (10mM Tris-HCl [pH 8.0], 60mM KCl, 1mM EDTA, 1mM dithiothreitol, proteinase inhibitors, and 0.3% NP-40). After 5 min on ice, the lysates were centrifuged at 1,000g at 4°C for 5 min, and the pelleted nuclei were washed in lysis buffer without NP-40. Each nuclear pellet was resuspended in an equal volume of nuclear extraction buffer (20mM Tris-HCl [pH 8.0], 420mM NaCl, 1.5mM MgCl$_2$, 0.2mM EDTA, and 25% glycerol), and NaCl was added to obtain a final concentration of 400 mM. After incubation at 4°C for 10 min, the nuclei were centrifuged at 25,000g for 5 min. The supernatant fraction was used as nuclear extract.

3.2. Electrophoretic mobility shift assay (EMSA)
3.2.1. Labeling probe with digoxigenin
1 nmol of each oligonucleotide (sense 5´-CGTGG GGCGC GGCCC TGAAT GCAGA GCCCG GCCTG TTTAC CTTGT GGCGG G-3´ and antisense 5´-CCCGC CACAA GGTAA ACAGG CCGGG CTCTG CATTC AGGGC CGCGC CCCAC G-3´) was annealed by heating at 95°C for 10 min and slowly cooled to room temperature in TEN-buffer [Tris (10mM, pH 8.0), EDTA (1mM, pH 8.0), and NaCl (100mM)]. The double-stranded oligonucleotide was then labeled using the reagents provided in the DIG Gel Shift Kit (Roche, Indianapolis, IN). One hundred nanograms of the fragment was added on ice to 4 µl 5× labeling buffer, 4 µl 25mM CoCl$_2$, 1 µl 1mM DIG-ddUTP, and 400 U terminal transferase in a final volume of 20 µl and incubated at 37°C for 1 hr. The DNA was then precipitated with 2 µl 4M LiCl and 60 µl 100% ice-cold ethanol and incubated at -70°C for 2 hr. The precipitated DNA was then pelleted by centrifugation for 15 min at 4°C, 13,000g, washed once with 100 µl ice-cold 70% ethanol and recentrifuged for an additional 15 min. After the pellets were air-dried, the DNA was resuspended in ddH$_2$O to a final concentration of 4 ng/µl. The efficiency of the labeling reaction was first tested using a dot blotting assay. Serial dilutions were spotted on a nylon membrane and detected. The resulting intensity was then directly compared to the control labeled oligonucleotide provided with the labeling kit. The probe was then stored at -20°C.

3.2.2. Binding reaction
Three micrograms of nuclear extraction were added to 0.8 ng of the digoxigenin-labeled probe. The 25-µl reaction mixture contained 1 µg poly d(I-C), 1 µg poly L-lysine, and 5 µl 5× binding buffer. For competition experiments, excess 8-ng unlabeled DNA was incubated with the reaction mixture for 15 min before the addition of the labeled probe. The mixture was electrophoresed at 10 V/cm through a 0.5× TBE- 6% polyacrylamide gel (acrylamide-bisacrylamide, 29:1). The gel was then transferred to a positively charged nylon membrane and the DNA cross-linked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA).

3.2.3. Detection
An amount of 10× blocking reagent (Roche) was prepared in a maleic acid buffer [0.1M maleic acid(pH 7.5), 0.15M NaCl] according to the manufacturer's instructions.

The nylon membrane was blocked for 30 min at room temperature in blocking reagent and then incubated with antidigoxigenin coupled to alkaline phosphatase diluted 1:10,000 for 30 min. The membrane was washed twice for 20 min each with 200 ml of maleic acid buffer with 0.3% Tween 20 added. After 5-min equilibration to pH 9.5 in 50 ml of the detection buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl), the membrane was carefully placed on a plastic sheet protector. The detection substrated, CDP-Star was diluted 1:100 in detection buffer, and 2 ml of diluted substrate was used to cover the membrane. After incubating 15 min, the membrane was exposed for 15 min.

3.3 Chromatin immunoprecipitation (ChIP)
In vivo binding of Sox9 to the 51-bp positive regulatory element (PRE) of the COMP promoter was investigated using the ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY). Confluent mouse fibroblast (NIH3T3) cells were transfected with plasmid encoding pFLAG-Sox9. The transfected cells were cultured on 10-mm dishes and then treated with formaldehyde (1% final concentration) to cross-link Sox9 to the DNA. Cells were washed with cold phosphate-buffered saline and lysed in SDS lysis buffer (1% SDS, 10mM EDTA, and 50mM Tris-HCl (pH 8.1)). The lysate was sonicated to shear DNA to a length between 200 and 1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2mM Tris-HCl (pH 8.1), and 150mM NaCl) and incubated with anti-Sox9 or control antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C with rotation. To collect DNA/protein complexes, salmon sperm DNA/protein A-agarose slurry was added to the mixture and incubated for 1 hr at 4°C with rotation, and the DNA/protein A-agarose was pelleted by centrifugation. After extensive washing of the pellet with a series of wash buffers, the pellet was dissolved with 250 µl of elution buffer and centrifuged to remove the agarose. The supernatant was treated with 20 µl of 5M NaCl and heated to 65°C for 4 hr to reverse the Sox9-DNA cross-link. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover the DNA. For PCR of the COMP promoter region using the chromatin-immunoprecipitated DNA, one-tenth of the DNA was PCR-amplified using forward primer 5´-tgcctttctctcctcaga-3´ and reverse primer 5´-actagaacgcaggcagtg-3´. Thirty-five cycles of PCR at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec were performed. PCR products were analyzed on 1% agarose gel.

3.4. Reporter gene assay
RCS cells grown to ~50% confluence in 60-mm culture dishes were transfected with 1 µg of reporter construct (-1925COMPluc, -125COMPluc, or -75COMPluc) (39, 40) along with 1 µg of pSVGal plasmid (internal control) and various amounts of mammalian expression plasmids encoding Sox9, Sox5, Sox6, CBP, and p3000, as indicated in Figs. 3, 5, 6 and 7. At 48 hr after transfection, the cultures were harvested and lysed. Luciferase assays were performed using 20 µl of cell extract and 100 µl of luciferin substrate (Promega, Madison, WI). β-Galactosidase assays were determined

A -125 CGTGGGGCGCGGCCCTG*AATGCA*GAGCCCGGCC*TGTTTA*C*CTTGTG*GCGGG -75
SBE1   SBE2   SBE3
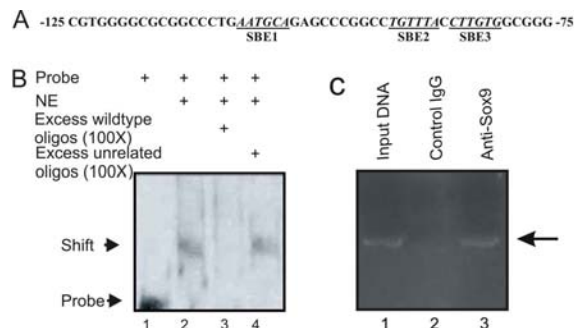
Figure 1. Sox9 binds to the PRE in the COMP promoter. (A) DNA sequence of 51-bp PRE (-125 to -75) of the COMP promoter. Putative Sox9-binding elements (SBEs) are underlined and italicized. (B) Sox9 binds to the PRE *in vitro*, assayed by EMSA. Nuclear extracts prepared from RCS cells transfected with pFLAG-Sox9 were incubated with DIG-labeled PRE probe in the reaction buffer (20 µl). For competition experiments, a 100-fold excess of wildtype PRE or unrelated DNA oligodeoxynucleotide was added. The reaction mixture was analyzed by gel electrophoresis; the positions of the Flag-Sox9/PRE complex ("Shift") and the free DNA probe ("Probe") are indicated. (C) Sox9 binds to the PRE of the COMP promoter in living cells, assayed by ChIP. 10T1/2 cells transfected with an expression plasmid pFlag-Sox9 were cross-linked by formaldehyde treatment and lysed. Cell lysates were subjected to immunoprecipitation with control IgG (lane 2) or anti-Sox9 antibodies (lane 3). Input DNA (lane 1, serving as positive control) and DNA recovered from the immunoprecipitation were amplified by PCR.

using a Beta-Galactosidase Assay Kit (Tropix, Foster City, CA) following the manufacturer's protocol. β-Galactosidase activity and luciferase activity were measured using a Mini-Lum luminometer (Bioscan, Washington, DC).

3.5. Generation and characterization of the constructs encoding small interfering RNA (siRNA) against Sox9 using pSUPER system

RNA target sequences for mouse Sox9 mRNA were selected using a siRNA target design online tool (www.oligoengine.com) and analyzed by a BLAST search to ensure gene specificity. The target sequences were 5´-AGAACTCCGGCTCCTACTA-3´ (I) and 5´-GAACGCACATCAAGACGGA-3´ (II). Oligonucleotides were synthesized and purchased from IDT Integrated DNA Technologies (Coralville, IA). Forward and reverse oligonucleotides were annealed to form a duplex. The annealed oligonucleotides were then ligated into the BglII–HindIII sites of the pSUPER vector to generate two constructs, pS-Sox9I and pS-Sox9II. To examine the efficiency of these two constructs in suppressing the expression of Sox9, C3H10T1/2 cells plated at a density of 3×10^5 cells/well in 6-well tissue culture were transfected with 2 µg of pSuper vector, pS-Sox9I, or pS-Sox9II together with 1 µg of Sox9 expression plasmid. After 48 hr, cell lysates were subjected to a western blotting assay with anti-Sox9 antibodies.

3.6. Real-time PCR assay

To investigate whether Sox9 transcriptional factor is required for COMP gene induction in the course of chondrogenesis, mesenchymal stem 10T1/2 cells grown to 50-80% confluence in 60-mm culture dishes were transfected with 10µg of either pSuper vector, pS-Sox9I, or pS-Sox9II and the micromass cultures of transfected cells were used for performing an *in vitro* chondrogenesis assay in the presence of 100 ng/ml recombinant bone morphogenetic protein 2 (BMP-2) (48). After 5 days, cultures were collected for measuring COMP gene expression using the TaqMan system with commercial primers and a probe for COMP (Applied Biosystems). To examine whether Sox9-mediated COMP gene activation is affected by Sox5 and Sox6 transcriptional factors and CBP and p300 coactivators, 10T1/2 or RCS cells were transfected with mammalian expression constructs encoding Sox9, Sox5, Sox6, CBP, p300, or their various combinations, as indicated in Figs. 6 and 7, and 48 hour later the COMP gene expression was examined by real-time PCR, as described above.

PCR was performed using sequence-specific primers and probe premix (Applied Biosystems, Foster City, CA) for mCOMP and mGAPDH (serving as internal control). PCR reactions for all samples were performed in optical tubes with 100 ng total RNA, 1 µl each primer and probe premix and 4 µl of TaqMan 5× buffer (Applied Biosystems) in a 20-µl reaction volume. The amplification reaction was carried out over 40 cycles with the following parameters: an initial holding stage of 2 min at 50°C, reverse transcription of 60 min at 60°C, deactivation of UNG of 10 min at 95°C, followed by a two-step cycling program of 15 sec melting at 95°C and 1 min annealing and extension at 62°C. Signal was detected by the ABI PRISM 7300 Sequence Detection System (Applied Biosystems).

An optimal real-time PCR condition was achieved through test runs by monitoring the growth curve. It was verified by the linear correlation between the logarithmic amount of mCOMP and mGAPDH cDNA in each sample and the cycle threshold (Ct). These conditions were then used in PCR reactions for all the cDNA samples. The Ct for both COMP and GAPDH were then measured and relative transcription levels were calculated.

4. RESULTS

4.1. Sox9 binds to the PRE in COMP promoter *in vitro*

The gene for COMP encodes a pentameric noncollagenous matrix protein that is expressed predominantly in articular cartilage. We have previously delineated a 30-bp negative regulatory element (-1775 to -1746, NRE) and a 51-bp positive regulatory element (-125 to -75, PRE) from COMP promoter. Subsequently we isolated LRF, a poxvirus and zinc finger (POZ) domain-containing transcriptional repressor, as a NRE-binding factor and established that LRF recruits histone deacetylase 1 (HDAC1) and inhibits COMP gene expression (48). Since there are three HMG-like sequences located in the PRE (-125 to -75) of COMP promoter, and one of them has been shown to associate with Sox9, a chondrocyte-specific transcription factor (Figure 1A). We first examined whether Sox9 is able to bind PRE in an electrophoretic mobility
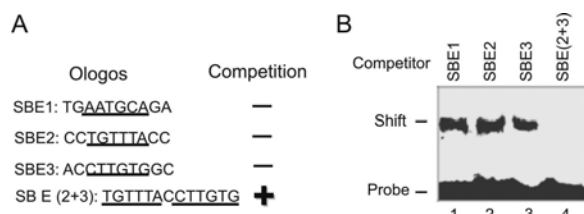
Figure 2. Identification of the Sox9 binding motif in the PRE of the COMP promoter. (A) Sequences of various oligodeoxynucleotides used for competition assays in (B). Putative Sox-binding element (SBE) is underlined. Competition effect of oligodeoxynucleotides on the binding of Sox9 to the PRE probes is indicated by "+" and "−". (B) EMSA with the same protein fraction and probes as in Figure 1B in the presence of excess of various oligodeoxynucleotides, as indicated.
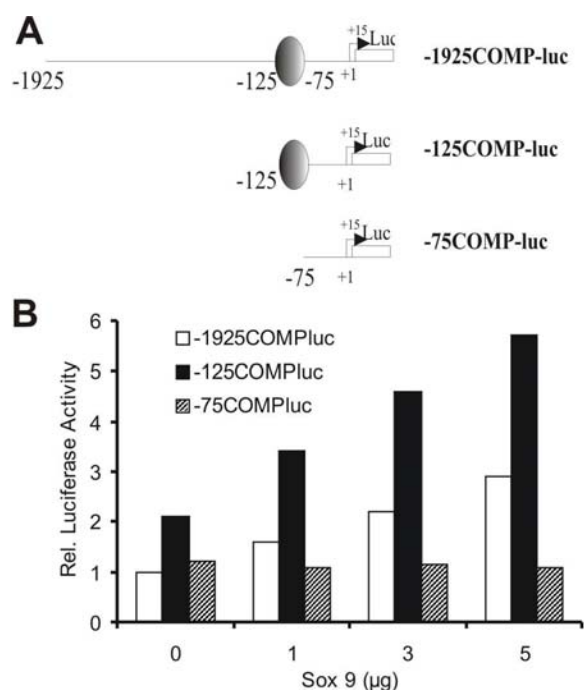
Figure 3. Activation by Sox9 of COMP-specific reporter constructs is PRE-dependent. (A) Schematic structures of three COMP-specific reporter constructs. The indicated segments from the 5´-flanking region of the COMP gene were linked to a DNA segment encoding luciferase (open boxes). Numbers indicate distances in nucleotides from the first nucleotide of intron 1. The PRE is represented by an oval. (B) Sox9 activates COMP-specific reporter construct activities. The indicated reporter gene was transfected into RCS cells with various amounts of pFLAG-Sox9 expression plasmid, as indicated, as well as a pSVgal internal control plasmid. At 48 hr after transfection, the cultures were lysed and β-galactosidase and luciferase activity determined. Activity of -1925COMPluc is arbitrarily set to 1 in the absence of Sox9.

shift assay (EMSA) (Figure 1B). Incubation of the digoxigenin-labeled PRE probe with the nuclear extracts

prepared from RCS cells transfected with the mammalian expression plasmid encoding Sox9 resulted in a specific Sox9·PRE complex (lane 2 in Figure 1B). The binding of probe to FLAG-Sox9 *in vitro* (lane 3) was completely competed by excess unlabeled probes (lane 3), but not unrelated oligodeoxynucleotide (lane 4), indicating that the binding of Sox9 to PRE is sequence-specific.

4.2. Sox9 binds to the PRE in the COMP gene promoter in living cells

To determine whether Sox9 also binds to PRE *in vivo*, we performed ChIP assays, which are important for defining interactions of factors with specific DNA elements in living cells. ChIP was carried out in NIH3T3 cells transfected with a mammalian expression plasmid encoding FLAG-tagged Sox9. After cross-linking with formaldehyde, cell lysates were immunoprecipitated with either control IgG (negative control) or anti-Sox9 antibodies, and the DNA purified from this coprecipitation was analyzed by PCR with PCR primers that spanned the PRE in the COMP promoter. As shown in Figure 1C, we observed a clear PCR product using DNA isolated from immunoprecipitated complexes with anti-Sox9 (lane 3) antibodies but not with control IgG (lane 2), indicating that the Flag-tagged Sox9 binds to the PRE of the COMP promoter in transfected living cells.

4.3. Identification of the Sox9-binding elements (SBEs) in the PRE

Once the interaction between Sox9 and the PRE became clear, we sought to identify the SBE in the 51-bp PRE by performing EMSA (Figure 2). The same probe as used in Figure 1B was incubated with nuclear extracts prepared from pFlag-Sox9-transfected RCS cells in the presence of various excess competitor oligonucleotides as indicated. The competition effects of the oligonucleotides tested on the binding of Sox9 to PRE is summarized in Figure 2A and shown in Figure 2B. SBE1 (TGAATGCAGA), SBE2 (CCTGTTTACC), and SBE3 (ACCTTGTGGC) had no effect on the binding of Sox9 to PRE; however, the 13-bp oligonucleotide spanning SBE2 and SBE3 totally abolished the interaction between Sox9 and PRE. These findings suggest that 13 nucleotides (TGTTTACCTTGTG) are required for the association of Sox9 and PRE in the COMP promoter.

4.4. Sox9 activates COMP-specific reporter construct activities in a PRE-dependent manner

To determine whether Sox9 activates transcription of the COMP promoter using reporter gene assays, three COMP-specific reporter gene plasmids, -1925COMPluc, -125COMPluc, and -75COMPluc, were generated in which segments with or without an PRE from the 5´-flanking region of the COMP gene were linked to the upstream end of a region encoding luciferase in the pGL2 basic vector (Figure 3A). We transfected RCS cells with these reporter constructs together with a Sox9 mammalian expression plasmid pFlag-Sox9. As shown in Figure 3B, Sox9 activated both COMP-specific reporter constructs that contained PRE, and the transactivations were dose-dependent. Interestingly, the shorter reporter gene (-125COMPluc) is more susceptible to Sox9 transfection,
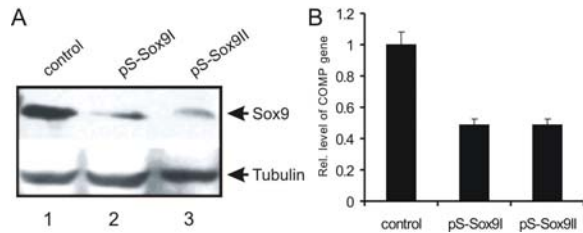
Figure 4. Sox9 is required for COMP gene expression in chondrogenesis. (A) Both pS-Sox9I and pS-Sox9II, which produce siRNA against Sox9, efficiently reduce the level of Sox9 protein, as determined by western blotting assay. C3H10T1/2 cells were transfected with Sox9 expression plasmid together with pSuper vector (lane 1), pS-Sox9I, or pS-Sox9II, and cell lysates were subjected to western blotting assay with either anti-Sox9 (top panel) or antitubulin (bottom panel, serving as internal control) antibodies. (B) Suppression of Sox9 by siRNAs inhibits COMP gene expression, assayed by real-time PCR. The micromass cultures of 10T1/2 cells transfected with either pSuper (control), pS-Sox9I, or pS-Sox9II, as indicated, were used for an *in vitro* chondrogenesis assay in the presence of 100 ng/ml of recombinant BMP-2, and endogenous COMP gene expression was determined by real-time PCR. Expression of COMP in each sample was normalized against the 18S rRNA endogenous control. The normalized values were then calibrated against the control value. The units are arbitrary, and the leftmost bar ("control") indicates a relative level of COMP of 1.

suggesting that the transactivation of the long reporter gene (-1925COMPluc) might be suppressed by transcriptional repressors, including LRF (48). In the case of the shortest COMP-specific reporter construct (-75COMPluc), which lacks the PRE, Sox9 expression did not show any effects on its activity, indicating that the transactivation of COMP gene expression depends on this 51-bp PRE located in the proximal region of the COMP promoter.

4.5. Suppression of Sox9 using the siRNA approach inhibits COMP gene expression

We next examined whether suppression of Sox9 will inhibit COMP gene expression. Two constructs (pS-Sox9I and pS-Sox9II) based on pSUPER RNAi system were generated and transfected together with a Sox9 expression plasmid into C3H10T1/2 cells. As shown in Figure 4A, both pS-Sox9I and pS-Sox9II markedly reduced the level of Sox9 protein. We then performed *in vitro* chondrogenesis with well-established BMP-2-treated microsmass cultures of mesenchymal stem 10T1/2 cells carrying either pSuper vector, pS-Sox9I construct, or pS-Sox9II construct, and COMP gene expression was determined with real-time PCR. As expected, COMP gene was induced during this process, but this induction was strongly suppressed by either pS-Sox9I or pS-Sox9II, which produce siRNA that specifically targets Sox9 (Figure 4B). These results demonstrated that Sox9, an essential transcriptional factor for chondrogenesis, is also required for COMP gene expression. These findings support the previous report that COMP gene expression is defective in Sox9-null mice (45).

4.6. Full activation of COMP gene expression requires a combination of Sox9, Sox5, and Sox6

Since COMP gene expression was found to be undetectable in Sox5; Sox6 double-null mice (60, 61), we next tested whether Sox5 and Sox6 are involved in COMP gene activation mediated by Sox9. We first transfected RCS cells with a COMP-specific reporter construct -1925COMPluc with various expression plasmids or combination. As shown in Figure 5A, although Sox9 alone activated the COMP-specific reporter construct (approximately 2- to 3-fold), the transactivation was strongly enhanced by cotransfection of either Sox5 (approximately 5- to 6-fold) or Sox6 (approximately 8- to 9-fold). More significantly, a combination of Sox9, Sox5, and Sox6 resulted in an approximately 65-fold increase in reporter gene activity compared with control. These findings suggest that full activation of Sox9-mediated COMP gene activity requires the involvements of Sox5 and Sox6 cofactors. To further investigate whether this is also true for endogenous COMP gene expression, we next conducted a real-time PCR assay with C3H10T1/2 cells. As shown in Figure 5B, 24 hr after transfection, Sox9 did not increase COMP gene expression, whereas cotransfection of Sox5 plus Sox6 led to a 6.73-fold increase in the level of COMP mRNA; after 48 hr, Sox9 alone gave rise to an approximately 2-fold increase and the combination of all three transcription factors produced a 41.6-fold increase, indicating that Sox5 and Sox6 are critical for the regulation of endogenous COMP gene expression by Sox9.

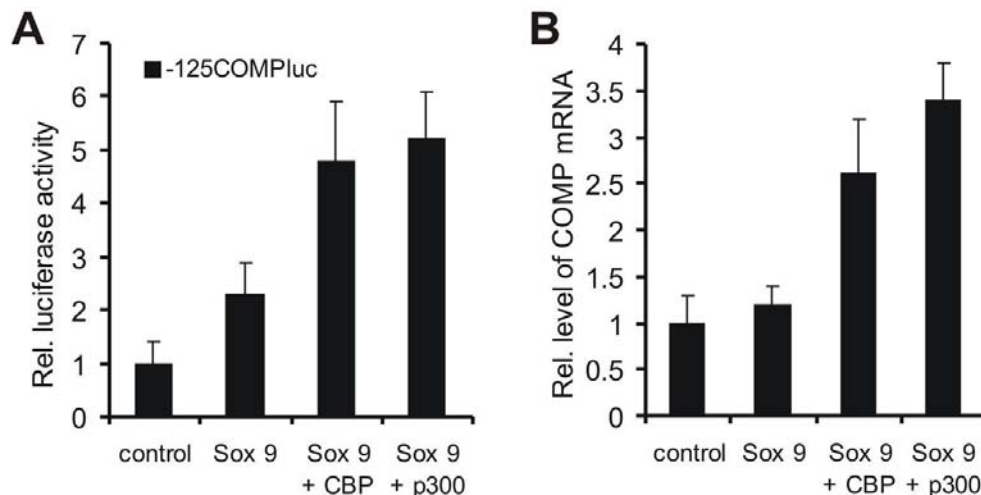### 4.7. Transactivation of COMP gene by Sox9 is enhanced by coexpression of CBP/p300 histone acetylase

It was recently reported that CBP and p300 associate with Sox9 and enhance Sox9-mediated transactivation by modification of chromatin structure through histone acetylase activity (25, 38, 64). We tested whether CBP/p300 also participate in COMP gene activation by Sox9 using the -125COMPluc reporter construct. CBP alone, p300 alone, and CBP plus p300 increased Sox9-mediated activation of the COMP-specific reporter construct by approximately 110%, 80%, and 150%, respectively. We also investigated whether this is also true for endogenous COMP gene expression by performing a real-time PCR assay with C3H10T1/2 cells. As revealed in Figure 6B, 24 hr after transfection Sox9 alone produced negligible effects on COMP mRNA level, whereas cotransfection of CBP or p300 resulted in approximately 2.7- and 3.4-fold increases in the of COMP mRNA, respectively. Take together, these findings indicate that both CBP and p300 are involved in the transactivation of COMP gene by Sox9.

4.8. Sox9 overcomes LRF-mediated inhibition of the COMP-specific reporter construct

Since LRF represses COMP gene expression (48), whereas Sox activates it (as shown in this study), we next determined whether there exists a balance between LRF and Sox9 in the control of COMP gene expression. We performed reporter gene assays with the COMP-specific construct -1925COMPluc in which both an LRF-binding NRE (-1775 to -1746) and a putative Sox9-binding
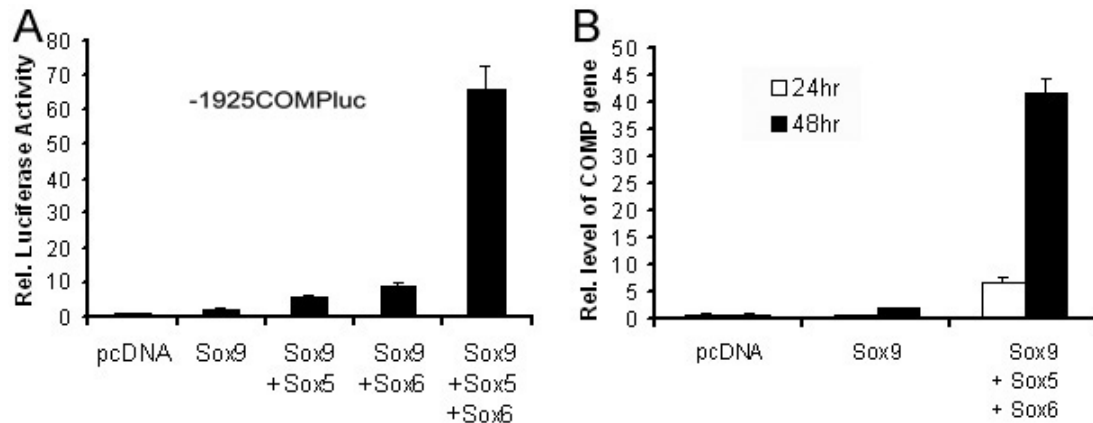
Figure 5. Activation of COMP gene expression by a combination of Sox9, Sox5, and Sox6 transcription factors. (A) Reporter gene assay. The COMP-specific reporter gene -1925COMPluc was transfected into RCS cells with empty vector or mammalian expression plasmids encoding Sox9, Sox5, Sox6, or combinations of them, as indicated, as well as a pSVgal internal control plasmid. At 48 hr after transfection, the cultures were lysed and β-galactosidase and luciferase activity determined. Activity of -1925COMPluc is arbitrarily set to 1 in the absence of Sox9. (B) Real-time PCR assays. 10T1/2 cells transfected with pCMV serving as a control), pFLAG-Sox9 or pFLAG-Sox9 plus pFLAG-Sox5 and pFLAG-Sox6 were cultured for 24 hr (open bar) or 48 hr (solid bar), and endogenous COMP gene expression was determined by real-time PCR. Expression of COMP in each sample was normalized against the 18S rRNA endogenous control. The normalized values were then calibrated against the control value. The units are arbitrary, and the leftmost bar indicates a relative level of COMP of 1.
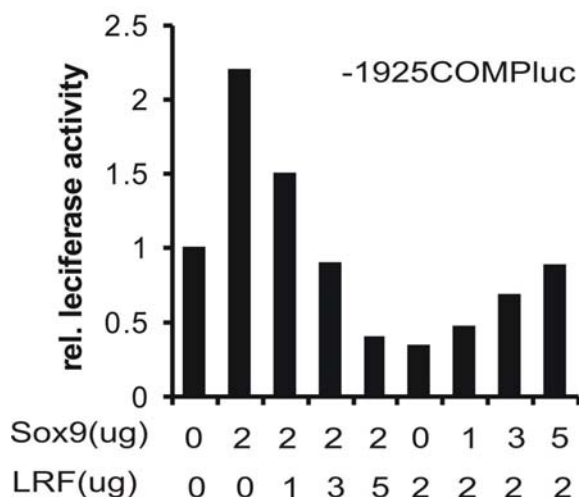

Figure 6. CBP/p300 enhancement of the Sox9-mediated transactivation of COMP gene expression. (A) Reporter gene assay. The COMP-specific reporter gene -1925COMPluc was transfected into RCS cells with empty vector or mammalian expression plasmids encoding Sox9, Sox9 plus CBP, or Sox9 plus p300, as indicated, as well as a pSVgal internal control plasmid ("control"). At 48 hr after transfection, the cultures were lysed and β-galactosidase and luciferase activity determined. Activity of -1925COMPluc is arbitrarily set to 1 in the absence of Sox9. (B) Real-time PCR assay. 10T1/2 cells transfected with pCMV ("control"), pFLAG-Sox9, pFLAG-Sox9 plus CBP, or pFLAG-Sox9 plus p300 were cultured 48 hr in the presence of 100 ng/ml of BMP-2, and endogenous COMP gene expression was determined by real-time PCR. Expression of COMP in each sample was normalized against the 18S rRNA endogenous control. The normalized values were then calibrated against the control value. The units are arbitrary, and the leftmost bar ("control") indicates a relative level of COMP mRNA of 1.

PRE (-125 to -75) are located. As shown in Figure 7, activation of this reporter gene activity by Sox9 was inhibited in the cotransfection of LRF, and similarly, excess exogenous Sox9 was able to overcome, at least in part, the LRF-mediated inhibition of the activity of this COMP-specific construct. This suite of assays suggests that the cross-talk between Sox9 activation and LRF repression complex is critical for the precise control of COMP gene expression in chondrogenesis and cartilage formation.

5. DISCUSSION

Our previous studies identified two regulatory elements in the 5′-flanking region of the murine COMP gene (39, 40): one is a 31-bp negative regulatory element (NRE) located between -1775 and -1746, to which a POZ-domain-containing transcription repressor LRF binds (48), and the other is a 51-bp positive regulatory element (PRE) located between -125 and -75 that contains three putative

Figure 7. Transactivation of Sox9 is abolished by the coexpression of LRF, and excess Sox9 overcomes LRF-mediated inhibition. The COMP-specific reporter gene -1925COMPluc was transfected into RCS cells with empty vector or various amounts of Sox9 and LRF expression plasmids, as indicated, as well as a pSVgal internal control plasmid. At 48 hr after transfection, cultures were lysed and β-galactosidase and luciferase activities determined. Activity of -1925COMPluc is arbitrarily set to 1 in the absence of Sox9 and LRF.

Sox9-binding elements. In the present study, we present evidence that Sox9, a critical transcriptional factor of cartilage matrix genes and chondrogenesis, binds directly to the PRE in the promoter of the COMP gene (Figs. 1, 2). We also present evidence that Sox9 activates COMP gene expression and that repression of Sox9 by the siRNA approach dramatically inhibits COMP gene induction in the course of chondrogenesis (Figs. 3, 4). The molecular mechanism underlying Sox9-mediated gene transactivation of the COMP gene appears to require the involvement of coactivators of Sox9, including Sox5, Sox6, and CBP/p300 (Figures 5, 6).

Although there exists three putative Sox9-binding elements, none of them could efficiently compete with the PRE probe for binding Sox9, and the 13 nucleotides (TGTTTACCTTGTG) that span the second and third Sox9-binding elements completely abolished the association of Sox9 and PRE (Figure 2). These results suggest that at least two Sox9-binding elements are required for the binding of Sox9 to COMP promoter. Other tissue-specific transcriptional factors, including skeletal muscle-specific MyoD transcriptional factor, were also found to require the existence of at least two MyoD-binding E boxes (47).

Sox9 is essential for the activation of cartilage matrix genes, including collagen II. Previous *in vivo* findings also demonstrated that Sox9 is required for COMP gene expression during development, since COMP gene expression is markedly downregulated in Sox9[flox/flox]; Col2a1-Cre mice -/- mice (45). Sox5 and Sox6 are the

downstream molecules of Sox9 and involves in the Sox9 – mediated chondrogenesis and COMP gene expression was hardly detectable in the Sox5;Sox6 double-null mice(60, 61). Using an siRNA approach, we demonstrated that Sox9 is indeed required for COMP gene activation; we further demonstrated that Sox9 directly binds to COMP promoter and activates COMP gene via associating with its cofactors, including Sox5 and Sox6. Although Sox9 appears to significantly activate COMP-specific reporter constructs (-1025COMPluc and -125COMPluc) (Figure 3), Sox9 alone had no marked effect on endogenous murine COMP gene expression; furthermore, robust expression of COMP gene was observed only in the presence of Sox5 and Sox6 (Figure 4), which is in line with the previous report that Sox9 alone failed to activate human COMP gene expression (56).

Sox9 has been reported to interact with several protein partners, and these interactions are important for its physiologic functions and subcellular localization and degradation. There exist physical and functional interactions between Sox9 and beta-catenin. Sox9 markedly inhibits activation of beta-catenin-dependent promoters and stimulates degradation of beta-catenin by the ubiquitination/proteasome pathway. Beta-catenin physically interacts through its armadillo repeats with the C-terminal transactivation domain of Sox9 (2, 26). Sox9 was reported to interact with PGC-1alpha, a peroxisome proliferator-activated receptor gamma coactivator, and PGC-1alpha was shown to act as a coactivator for Sox9 during chondrogenesis (41). In a yeast two-hybrid screen PIAS1 (protein inhibitor of activated STAT-1), -3, -xalpha, and -xbeta were identified as Sox9-associated proteins. These PIAS proteins were shown to interact directly with SOX9 and control its function via affecting the cellular concentration and sumoylation of Sox9 (28). More importantly, Sox9 was found to associate with coactivators CREB-binding protein (CBP) and p300, and these interactions are critical for the control of some cartilage matrix genes, including collagen II and cartilage-derived retinoic acid-sensitive protein (CD-RAP) (25, 38, 41, 64). Our finding that CBP/p300 significantly enhance COMP gene activation by Sox9 also supports the notion that CBP and p300 are coactivators of Sox9 in the regulation of cartilage matrix genes.

It has long been established that histone acetylation is associated with transcriptional activity in eukaryotic cells (3, 55). It was subsequently demonstrated that acetylated core histones preferentially associate with transcriptionally active chromatin (29, 58, 66). Acetylation occurs at lysine residues on the amino-terminal tails of the histones, thereby neutralizing the positive charge of the histone tails and decreasing their affinity for DNA (34). As a consequence, histone acetylation alters nucleosomal conformation (54), which can increase the accessibility of transcriptional regulatory proteins to chromatin templates (44, 65). These observations suggest that histone acetylation can result in increased transcriptional activity *in vivo*. In accordance with this general correlation, the histone acetylases are typically associated with transcriptional activation, whereas histone deacetylases are
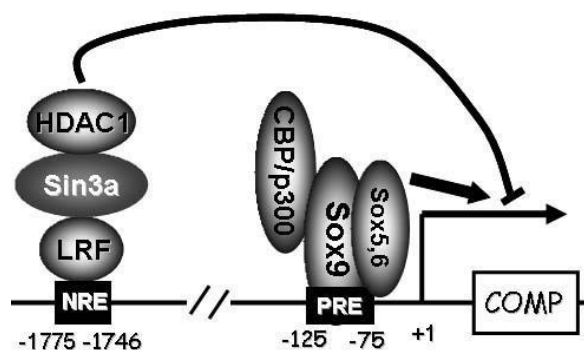
Figure 8. A proposed model for explaining the transcriptional control of COMP gene expression. Numbers indicate distances in nucleotides from the first nucleotide of intron 1. "→" and "⊢"indicate activation and repression, respectively.

typically associated with transcriptional repression. We previously reported that the transcription repressor LRF associated with histone deacetylase, and in this study we demonstrated that the histone acetylase CBP/p300 is involved in the Sox9- mediated activation of COMP gene; furthermore, there exists a balance between LRF repressor and Sox9 activator in the control of COMP gene expression (Figure 7). Based on these findings and the literature, we propose a model for explaining the transcriptional control of COMP gene expression (Figure 8). In an undifferentiated state, LRF binds to the NRE (-1775 to-1746) located in the distal region of the COMP promoter and recruits histone deacetylase 1 (HDAC1); this interaction may be bridged by the Sin3A corepressor, which has been found to bind both LRF (63) (also our unpublished data) and HDAC1 (10, 27). When the LRF/Sin3A/HDAC1 repressor complex associates with the COMP gene promoter, chromatin is condensed and the COMP gene is silenced. When mesenchymal tissues are triggered by extracellular signals for differentiation, however, the repressor complex disassociates from the COMP promoter, and the SOX9/CBP/p300 activator complex (23, 64) binds to the PRE (-125 to -75) in the proximal region of the COMP promoter, which results in loosening of the chromatin structure and activation of COMP gene expression. Although the details of the mechanism remain unclear, it is speculated that Sox5 and Sox6 associate with Sox9/CBP/p300 and form a ternary activation complex. It is conceivable that in addition to the cofactors shown in Figure 8, other unidentified LRF- or Sox9-binding cofactors positively or negatively regulate LRF and Sox9 activity by influencing (1) their DNA-binding activity; (2) the formation of repressor and activator complexes; (3) the posttranscriptional modifications of LRF and Sox9; and/or (4) the shuttling between nucleus and cytoplasm of Sox9 and LRF. It is speculated that Sox9- and LRF-mediated protein-protein interactions are also important in the precise control of COMP gene expression and chondrogenesis.

6. ACKNOWLEDGMENTS

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Abbreviations: BLAST, basic local alignment search tool;
CBP, CREB-binding protein; CD-RAP, cartilage-derived
retinoic acid–sensitive protein; ChIP, chromatin
immunoprecipitation; COMP, cartilage oligomeric matrix
protein; DIG, digoxigenin; EMSA, electrophoretic mobility
shift assay; HDAC1, histone deacetylase 1; LRF,
leukemia/lymphoma-related factor; NRE, negative
regulatory element; PRE, positive regulatory element; RCS,
rat chondrocsarcoma; PGC-1, peroxisome proliferator-
activated receptor gamma coactivator; PIAS1, protein
inhibitor of activated STAT-1; SBE, Sox9-binding
element;Sox9, SRY-related high-mobility group box gene
9; POZ, a poxvirus and zinc finger; ARF, Alternative
reading frame protein

Key Words: Sox9, COMP, Sox6, Sox5, CBP, p300

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