MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 2336–2346 Vol. 17, No. 4
0270-7306/97/$04.00+0

SOX9 Is a Potent Activator of the Chondrocyte-Specific Enhancer of the Proα1(II) Collagen Gene

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Received 26 November 1996/Returned for modification 19 December 1996/Accepted 9 January 1997

The identification of mutations in the SRY-related *SOX9* gene in patients with campomelic dysplasia, a severe skeletal malformation syndrome, and the abundant expression of *Sox9* in mouse chondroprogenitor cells and fully differentiated chondrocytes during embryonic development have suggested the hypothesis that SOX9 might play a role in chondrogenesis. Our previous experiments with the gene (*Col2a1*) for collagen II, an early and abundant marker of chondrocyte differentiation, identified a minimal DNA element in intron 1 which directs chondrocyte-specific expression in transgenic mice. This element is also a strong chondrocyte-specific enhancer in transient transfection experiments. We show here that *Col2a1* expression is closely correlated with high levels of *SOX9* RNA and protein in chondrocytes. Our experiments indicate that the minimal *Col2a1* enhancer is a direct target for Sox9. Indeed, SOX9 binds to a sequence of the minimal *Col2a1* enhancer that is essential for activity in chondrocytes, and SOX9 acts as a potent activator of this enhancer in cotransfection experiments in nonchondrocytic cells. Mutations in the enhancer that prevent binding of SOX9 abolish enhancer activity in chondrocytes and suppress enhancer activation by SOX9 in nonchondrocytic cells. Other SOX family members are ineffective. Expression of a truncated SOX9 protein lacking the transactivation domain but retaining DNA-binding activity interferes with enhancer activation by full-length SOX9 in fibroblasts and inhibits enhancer activity in chondrocytes. Our results strongly suggest a model whereby SOX9 is involved in the control of the cell-specific activation of *COL2A1* in chondrocytes, an essential component of the differentiation program of these cells. We speculate that in campomelic dysplasia a decrease in SOX9 activity would inhibit production of collagen II, and eventually other cartilage matrix proteins, leading to major skeletal anomalies.

Acquisition of the chondrocyte phenotype is one of the major pathways of mesenchymal cell differentiation. During and after condensation of mesenchymal cells, cartilage-specific genes are switched on (2, 12). The products of these genes, which include collagen types II, IX, and XI, the link protein, and aggrecan, form the characteristic extracellular matrix of cartilages. In recent years, differentiation of mesenchymal cells into myocytes and adipocytes has been shown to be controlled by cell-specific transcription factors belonging to different protein families (10, 28, 39, 41, 46). By analogy, we speculate that specific transcription factors could also control the differentiation of mesenchymal cells into chondrocytes and activate cartilage-specific genes.

In order to identify transcription factors which control chondrocyte differentiation, we have used the gene for type II collagen (*Col2a1*), an early and abundant marker of chondrocytes (3), and have delineated a minimal sequence in this gene that is sufficient to direct chondrocyte-specific expression both in transgenic mice and in transient transfection experiments (35, 40, 55). Transfections were done in rat chondrosarcoma (RCS) cells, in primary chondrocytes, and in 10T1/2 fibroblasts and C_2C_{12} myoblasts. RCS cells are a unique cell line which stably expresses a fully differentiated chondrocyte phenotype (40). In transgenic-mouse embryos, four tandem copies of a 48-bp fragment of *Col2a1* intron 1 directed high-level chondrocyte-specific expression in all cartilages. This 48-bp element was also a strong enhancer in transient expression experiments in RCS cells and primary chondrocytes, but not in fibroblasts. This 48-bp element contains two inverted repeats of 11 bp each with one mismatch. Multiple copies of an 18-bp element that included the 3′ repeat were a potent enhancer in RCS cells and chondrocytes, but not in fibroblasts, and were able to direct chondrocyte expression in transgenic mice. In contrast, a multimer of an element containing the 5′ repeat was inactive.

Recent reports have suggested that SOX9 might play a role in chondrogenesis. SOX9 is a member of a large family of proteins which harbor a DNA-binding domain with >50% similarity to that of sex-determining region Y (SRY), the testis-determining gene in mammals (18, 29, 52). This DNA-binding domain is encoded by a variant of the HMG box (the acronym SOX is derived from the term SRY-type HMG box) first identified in the genes for high-mobility-group (HMG) proteins, a class of architectural components of chromatin (1, 19, 32). SRY and SOX proteins bind to (A/T)(A/T)CAA(A/T)G motifs in the minor groove of the DNA helix and induce DNA bending (5, 6, 21, 24). Several SOX proteins, including SOX9, are able to act as transcriptional activators through multimerized elements containing a consensus DNA-binding site for HMG domain proteins (24, 42, 48, 51); in experiments in which SOX9 was fused to the yeast GAL4 DNA-binding domain, the transcriptional activation domain of SOX9 was mapped to its C terminus (42).

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Mutations in and around *SOX9* cause campomelic dysplasia (14, 31, 49, 50). In this rare human skeletal malformation syndrome (25, 38), patients exhibit severe anomalies in multiple skeletal elements, including shortened and angulated long bones, hypoplastic scapulae, deformed pelvis and spine, a small thoracic cage, micrognathia, cleft palate, and a flat nasal bridge. Death frequently occurs soon after birth because of respiratory distress. The disease is also associated with nonskeletal anomalies and is frequently accompanied by autosomal XY sex reversal. In all but one case studied, mutations occurred in a single allele, consistent with a dominant mode of inheritance of the disease. A majority of mutations identified were predicted to interfere with or abolish the normal function of SOX9, suggesting that the disease was due to haploinsufficiency. In a few cases, chromosomal translocations were identified 50 kb or more 5′ to the SOX9 gene.

During mouse embryonic development, *Sox9* is expressed at high levels at all sites where cartilage is being laid down (53). Abundant *Sox9* expression becomes evident in mesenchymal condensations before overt chondrocyte differentiation and persists during cartilage deposition. Hence, the pattern of expression of *Sox9* suggests that Sox9 might be causally related to chondrocyte differentiation and have a fundamental role in the formation of all cartilages. This hypothesis is supported by the existence of anomalies in all cartilage-derived skeletal structures in those with campomelic dysplasia. High levels of SOX9 are also found in the testis throughout development, specifically in Sertoli cells, which could account for the high frequency of sex reversal in XY patients with campomelic dysplasia, and this led to the suggestion that SOX9 could also play a role in the differentiation of these cells (9, 26). *Sox9* is also expressed at low levels in some noncartilaginous tissues, suggesting that it may have additional roles in development (49, 50).

In this study, we asked whether SOX9 might contribute to the control of *Col2a1* expression in chondrocytes. We show that there is an excellent correlation between expression of *Col2a1* and *Sox9* in chondrogenic cells, that SOX9 binds directly to the *Col2a1* enhancer at a site which is essential for chondrocyte-specific expression, and that forced expression of SOX9 is sufficient to activate this enhancer at high levels in nonchondrogenic cells. These results strongly suggest that SOX9 plays a crucial role in the specific activation of *COL2A1* in chondrocytes and raise the possibility that SOX9 might be a key transcriptional regulator of chondrocyte differentiation.

MATERIALS AND METHODS

Cell types and cultures. RCS cells, newborn mouse rib chondrocytes and skin fibroblasts, ROS 17/2.8 cells, MC615 cells, and MCTs cells were obtained and cultured as described previously (34, 35, 37, 40). Other cell lines were from the American Type Culture Collection (Rockville, Md.). The subline 714 was used for BALB/3T3 fibroblasts (17).

RNA analysis. Total RNA, Northern blots, and DNA probes were prepared as described previously (40). The *Sox9* probe was a 255-bp *Nar*I fragment of the mouse cDNA corresponding to nucleotides 1070 to 1324 (53). The 18S rRNA probe was from Ambion (Austin, Tex.).

Isolation and characterization of SOX9 antibodies. Rabbit antibodies directed against SOX9 were obtained by using a peptide (HSPQHWEQPVYTQLT) corresponding to the C terminus of human SOX9 and were purified as described previously (9). In Western blots of the products of in vitro transcription-translation directed by SOX9 cDNA, these antibodies recognized only the major product of this reaction; no polypeptides were recognized by these antibodies in Western blots of control reticulocyte lysates. In addition, in Western blots of total lysates from various cell types, these antibodies recognized only one polypeptide (see Fig. 1C), whose electrophoretic mobility was identical to that of SOX9 synthesized in vitro (data not shown) and whose relative abundance correlated with *Sox9* RNA levels in these cells. These experiments demonstrated the high specificity of these antibodies for SOX9.

Western blotting. Cell lysates were prepared as described previously (7), and Western blotting was done with the ECL kit from Amersham (Arlington Heights, Ill.). Mouse anti-FLAG M2 antibodies were purchased from IBI-Kodak (Rochester, N.Y.) and used at a dilution of 1:1,000. SOX9 antibodies were used at a 1:5,000 dilution.

Immunofluorescence. Mouse chondrocyte monolayers were treated with bacterial collagenase B (3 mg/ml in Dulbecco's modified Eagle's medium; Boehringer Mannheim) for 2 h at 37°C and washed in phosphate-buffered saline before being replated for a 2-day culture. The medium was supplemented with 50 μg of ascorbic acid per ml for the last 15 h, together with 1 mM cysteine and pyruvate. RCS and 10T1/2 cells were passaged and cultured under standard conditions for 2 days. Cell monolayers were fixed with methanol for 10 min and incubated in blocking buffer (phosphate-buffered saline with 5% goat serum and 3% bovine serum albumin) for 30 min. Incubation with primary antibodies was done for 1 h at room temperature in blocking buffer with rabbit SOX9 antibody diluted 1:500 and mouse monoclonal type II collagen antibody (2B1; provided by R. Mayne, University of Alabama, Birmingham) diluted 1:50. Secondary antibodies (Jackson ImmunoResearch, West Grove, Pa.) were fluorescein-conjugated goat anti-rabbit immunoglobulin G and Texas red-conjugated donkey anti-mouse immunoglobulin G, each diluted 1:200 in blocking buffer. Incubation with these antibodies was for 1 h in the presence of 5 μg of the DNA dye 4′,6-diamidino-2-phenylindole (DAPI) per ml. Slides were mounted with Aqua-Poly Mount (Polysciences, Warrington, Pa.).

Mammalian expression plasmids. Coding sequences for several SOX proteins were cloned into two modified versions of the pcDNA3.1(+) plasmid (Invitrogen, San Diego, Calif.), called pcDNA-5′UT and pcDNA-5′UT-FLAG. The pcDNA-5′UT plasmid was obtained by cloning between the *Hin*dIII and *Bam*HI cleavage sites of pcDNA3.1(+) a double-stranded oligonucleotide containing (from 5′ to 3′) a *Hin*dIII site, the proximal 54 bp of the 5′ untranslated leader of the herpes simplex virus *tk* gene (43), an ATG translation initiation codon, and a *Bam*HI site. pcDNA-5′UT-FLAG was obtained by cloning in the *Bam*HI site of pcDNA-5′UT a double-stranded oligonucleotide containing (from 5′ to 3′) a *Bgl*II site, the 24 bp of the FLAG sequence (23), and a *Bam*HI site. An almost-full-length coding sequence for human SOX9 (from codon 27, which directly follows the first ATG codon associated with a Kozak sequence, up to 39 bp of 3′ untranslated sequence) was made by PCR with primers that added a *Bam*HI site at the 5′ end and an *Xba*I site at the 3′ end of the reaction product and with a full-length *SOX9* cDNA clone as template (14). The PCR product was digested with *Bam*HI and *Xba*I and cloned in the two modified expression vectors. A digestion with *Bam*HI and *Sma*I was also performed to clone a truncated cDNA for *SOX9* (tr.SOX9; encoding amino acids 27 to 304). In the tr.SOX9-pcDNA vectors, a TAG sequence in the *Xba*I site of the plasmid served as the translation stop codon. Vectors encoding full-length mouse Sox4 and Sox5 were generated in the same way with full-length cDNAs as templates (11, 48). The identities of all PCR products were verified by DNA sequencing. A pCDMA vector (Invitrogen) encoding the full-length human SOX9 was also used and gave results similar to those obtained with SOX9-pcDNA-5′UT vectors (data not shown). A CBF-B-FLAG-pcDNA1 plasmid was used as internal control in some experiments (see Fig. 4). This plasmid encoded the B subunit of the CCAAT-binding factor CBF with a FLAG epitope at the C terminus (26a).

Synthesis of proteins in vitro and preparation of nuclear extracts. SOX proteins were synthesized by in vitro transcription-translation with the expression vectors described above and a single-tube protein system from Novagen, Inc. (Madison, Wis.). Nuclear extracts from RCS and BALB/3T3 cells were prepared as described previously (35).

Electrophoretic mobility shift assays (EMSAs). The 18-bp *Col2a1* enhancer probe and mutants were previously described (35) with the exception of the m(12-13) probe, which was synthesized following the same scheme but with nucleotides 12 and 13 mutated to A and C. The 48-bp enhancer probe was flanked at the 5′ and 3′ ends by *Bam*HI- and *Bgl*II-cleaved sites. The HMG probe, which was made of complementary oligonucleotides (5′-ggACACTGAG AACAAAGCGCTCTCACAC-3′ and 5′-ggGTGTGAGAGCGCTTTGTTCTC AGTGT-3′), corresponded to a fragment of the *CD3*ε enhancer (uppercase letters) that contains a consensus binding site for HMG box proteins (20); G residues (lowercase letters) were added at the 5′ ends for labeling. Protein-DNA binding reactions were carried out as previously described (35). Assays with nuclear extracts were performed with 10 μg of protein and 1 μg of poly(dG-dC) · poly(dG-dC). In vitro-synthesized proteins were assayed in the presence of 0.1 μg of herring sperm DNA. In supershift experiments, 0.1 μl of purified SOX9 antibody was added to each reaction mixture 15 min before addition of the DNA probe.

DNA bending experiments. The 18- and 48-bp *Col2a1* enhancer probes and the HMG probe were cloned in the *Xba*I site of the circular permutation vector pBend2 (27). Circularly permuted probes were prepared by cleavage with restriction enzymes (see Fig. 4A), ^{32}P end labeling, and purification by polyacrylamide gel electrophoresis. EMSAs were performed as described previously (35) with SOX9 synthesized by in vitro transcription-translation and 0.1 μg of poly(dG-dC) · poly(dG-dC). Bending parameters were calculated according to the method of Thompson and Landy (44).

Col2a1-luciferase constructions. All Col2a1-luciferase constructions but those containing a 100-bp intron 1 fragment (30) were described previously (35). Each construction contained a minimal mouse *Col2a1* promoter (nucleotides −89 to +6). Enhancer segments were cloned as one copy or several tandem copies directly upstream of the promoter. The 100-bp enhancer fragment was obtained

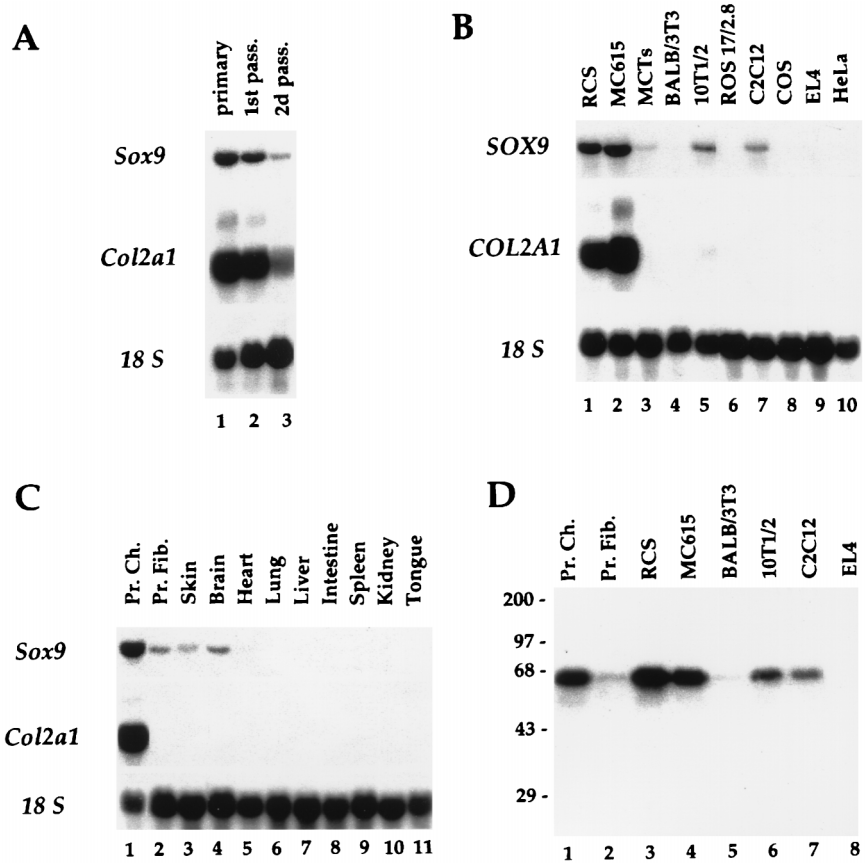

FIG. 1. Expression of *SOX9* and *COL2A1* in various cell types and tissues. (A) Northern blots of RNA from chondrocytes. Newborn mouse rib chondrocytes were cultured in a monolayer for 1 week (primary culture) and at the first (1st pass.) and second (2d pass.) passages. Filters containing about 10 μg of total RNA for each sample were hybridized with *Sox9* and *Col2a1* probes and with a probe for 18S rRNA, which was used as a reference for RNA loading. The size of the *Sox9* mRNA was about 4.5 kb. The decreases in *Sox9* and *Col2a1* RNAs were estimated to be at least 10-fold and almost 100-fold, respectively, by the second passage. (B) Northern blots of RNA from various cell lines. The following cell lines were analyzed at confluence: RCS cells, mouse MC615 and MCTs immortalized chondrocytes (34), mouse embryo BALB/3T3 and 10T1/2 fibroblasts, rat osteosarcoma ROS 17/2.8 cells, mouse C_2C_{12} myoblasts, monkey kidney COS cells, mouse lymphoma EL4 cells (these cells were grown in suspension), and human carcinoma HeLa cells. RNAs were analyzed as described for panel A. Note that very low levels of *Col2a1* RNA were occasionally found in 10T1/2 cells. (C) Northern blots of RNA from mouse tissues. Samples containing about 10 μg of total RNA from primary chondrocytes (Pr. Ch.), primary skin fibroblasts (Pr. Fib.), or various newborn mouse tissues were analyzed as described for panel A. (D) Western blot of SOX9 protein accumulated in various cell types. Two-microgram samples of protein from total cell lysates of primary chondrocytes (Pr. Ch.), primary skin fibroblasts (Pr. Fib.), and different cell lines were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and incubated with SOX9 antibodies. Protein standard M_rs are indicated on the left.

as a double-stranded oligonucleotide with a *Bam*HI site at the 5′ end and a *Bgl*II site at the 3′ end. After cloning, the absence of mutations was verified by DNA sequencing.

Transient transfections. COS cells were transfected by DNA-calcium phosphate coprecipitation (4). All other cell types were transfected either by electroporation as described before (35) or by using lipofectamine according to the instructions of GIBCO-BRL. Briefly, 5 μl of lipofectamine was mixed with a total of 2 μg of plasmid DNA in 200 μl of Opti-MEM medium (GIBCO-BRL). The mixture was preincubated for about 30 min, diluted to 1 ml in medium, and added to preestablished monolayers of 2.5×10^5 cells per 10-cm^2 dish for 5 h. Cells were further cultured for about 48 h in standard culture medium without antibiotics. Luciferase reporter plasmids and the pSV2βgal plasmid, used as an internal control for transfection efficiency, were cotransfected in a 3:1 ratio. Expression plasmids were cotransfected as indicated in the figure legends. Luciferase and β-galactosidase activities were assayed as described previously (35). Promoter activities were expressed as 10^3 luciferase units per β-galactosidase unit.

RESULTS

The high expression level of *SOX9* correlates with expression of *COL2A1* in chondrocytic cells. In newborn mouse primary chondrocytes, which were fully differentiated (33), *Sox9* and *Col2a1* mRNA levels were high (Fig. 1A, lane 1). The levels of both RNAs decreased in parallel as the cells dedifferentiated with time in culture (lanes 2 and 3). High levels of *Sox9* RNA were also associated with high levels of *Col2a1* RNA in highly differentiated RCS cells (40) and in the chondrogenic MC615 cell line (37) (Fig. 1B, lanes 1 and 2). In the other cell lines studied, which did not express *COL2A1*, *SOX9* RNA either was absent or was present at significantly lower levels (lanes 3 to 10). Levels of *Sox9* RNA in various newborn mouse tissues and primary skin fibroblasts were considerably lower than those in fully differentiated chondrocytes (Fig. 1C). In primary chondrocytes and skin fibroblasts and in the different cell lines that were examined, the levels of SOX9 protein varied in proportion with the levels of *SOX9* RNA (Fig. 1D). In immunofluorescence experiments with partially dedifferentiated chondrocyte cultures, the signal for Sox9 was strong in differentiated chondrocytes, which expressed type II collagen, and much weaker or negative in cells that did not express type II collagen (Fig. 2A to C). The latter cells presumably correspond to dedifferentiated chondrocytes and nonchondrocytic cells contaminating chondrocyte preparations. Sox9 was confined to the nucleus in both type II collagen-positive and -negative cells. Sox9 signal was strong in RCS cells (Fig. 2D), weak in

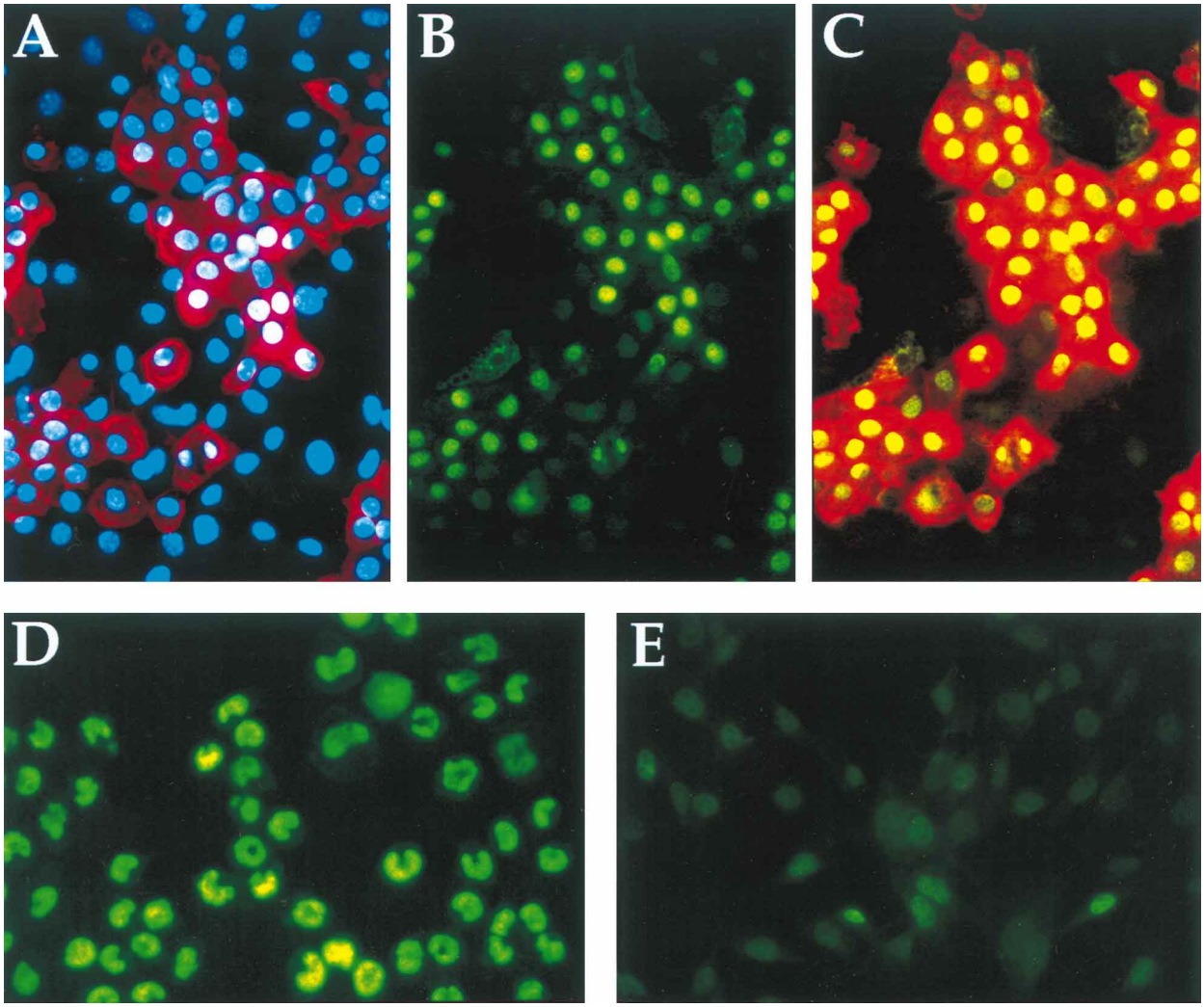

FIG. 2. Immunolocalization of Sox9 and type II collagen in cultured cells. (A to C) Mouse chondrocytes at the second passage, photographed in the same field in all three panels. (A) Staining of cell nuclei with DAPI in blue and immunostaining of type II collagen in red. (B) Immunostaining of Sox9 in green. Note that in a few type II collagen-negative cells, Sox9 signal is cytoplasmic. This is presumably due to an artifact since it was also observed in the absence of Sox9 antibody. (C) Immunostaining of Sox9 in green and of type II collagen in red. Note that although Sox9 is essentially nuclear and type II collagen is basically cytoplasmic and extracellular, overlapping of the two signals (yellow) is due to the fact that the staining was performed in whole-mount cultures. (D) Immunostaining of Sox9 in RCS cells. (E) Immunostaining of Sox9 in 10T1/2 fibroblasts.

10T1/2 fibroblasts (Fig. 2E), and predominantly nuclear in both cell types. Overall, the results of Fig. 1 and 2 indicated that expression of *COL2A1* correlated with high levels of *SOX9* RNA and protein. This close correlation was most clearly illustrated when individual cells were examined in cultures of chondrocytes.

SOX9 binds to the minimal *Col2a1* enhancer sequence. SOX9 was synthesized by in vitro transcription-translation as an almost-full-length protein (amino acids 27 to 509) or as a truncated protein (tr.SOX9; amino acids 27 to 304) which retained the HMG domain (amino acids 104 to 182) but lacked the transcription activation domain (amino acids 402 to 509) (14, 42). In EMSAs, SOX9 and tr.SOX9 formed complexes of similar intensity with a probe containing a consensus binding site for HMG domain proteins (the HMG probe) and with probes containing the 18- and 48-bp *Col2a1* enhancer elements (Fig. 3A).

Sox9 present in RCS cells bound to the 18-bp probe, since antibodies against SOX9 supershifted a complex formed between RCS cell nuclear extracts and the 18-bp probe that migrated at the same rate as the complex formed with SOX9 made in vitro (Fig. 3B). Binding occurred when poly(dG-dC) was used as a nonspecific competitor, but it was inhibited by poly(dI-dC) (data not shown). This result was in agreement with the observation that HMG domain proteins contact several A-T pairs in the minor groove of the DNA helix; indeed, I-C pairs mimic T-A pairs in the minor groove, whereas G-C pairs do not (8, 47). No such complex and no supershift occurred with BALB/3T3 extracts, which do not contain Sox9 (Fig. 3B).

Mutations of nucleotides 8 and 9, 10 and 11, 12 and 13, and 14 and 15 in the 18-bp element strongly decreased binding of SOX9, whereas mutations in the 7 nucleotides located at the 5′ end or in the 5 nucleotides located at the 3′ end did not affect binding of SOX9 (Fig. 3C). SOX9 thus bound to the CATTC ATG sequence in the middle of the 18-bp enhancer. This sequence contains 6 of 7 nucleotides of the consensus binding site defined for HMG proteins (C[T/A]TTG[T/A][T/A]) (24).

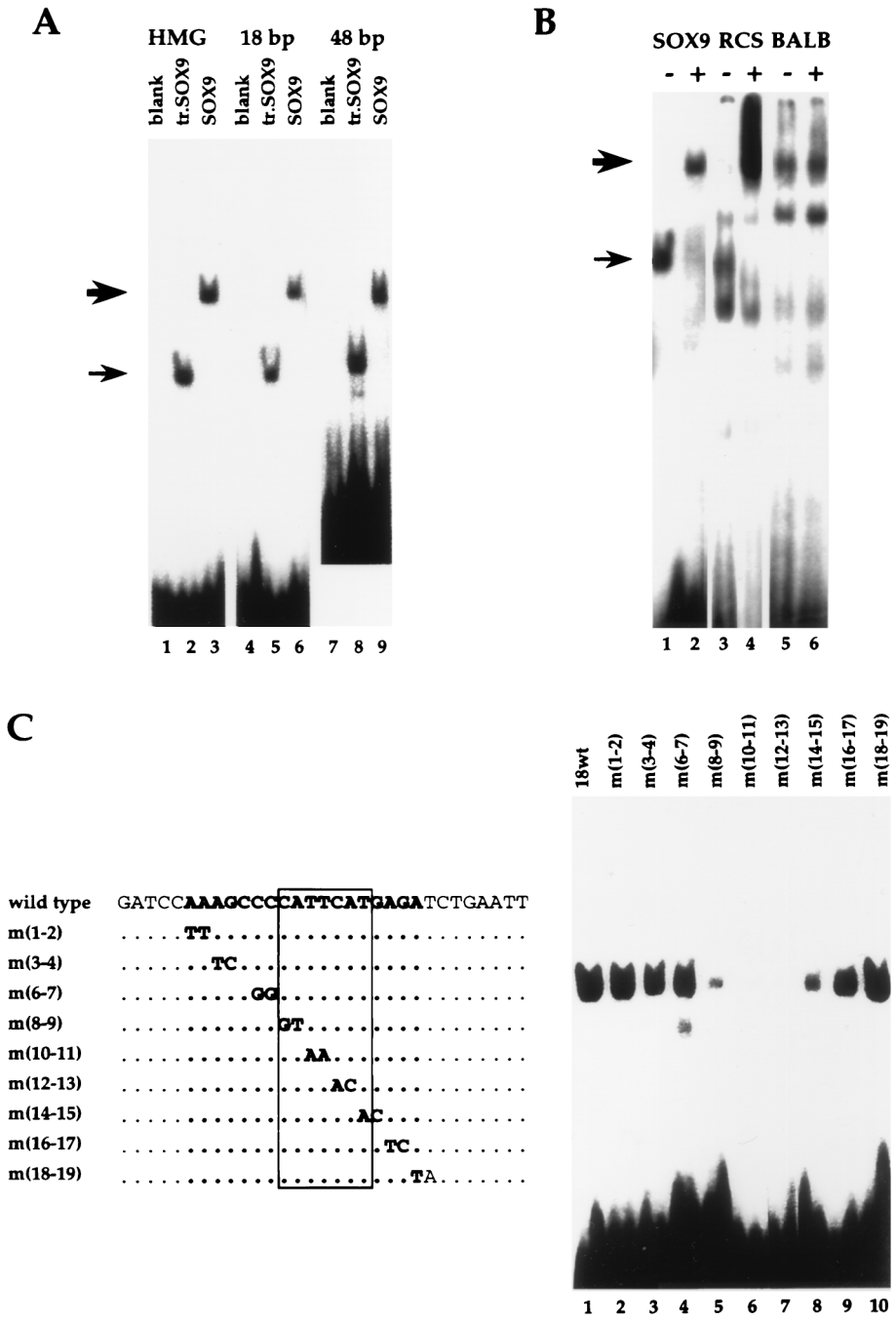

FIG. 3. Binding of SOX9 to the *Col2a1* enhancer. (A) Comparison between the binding of SOX9 to a consensus binding site for HMG domain proteins and its binding to the 18- and 48-bp *Col2a1* enhancer elements. EMSA was performed with products of in vitro transcription-translation from pcDNA-5′UT-FLAG plasmids encoding no protein (blank), truncated SOX9 (tr.SOX9), or almost-full-length SOX9 (SOX9). The specific activities of the HMG and the 18- and 48-bp labeled probes were equalized by dilution with unlabeled probe. The thin and thick arrows indicate the complexes with DNA of tr.SOX9 and SOX9, respectively. (B) Binding of Sox9 present in RCS cells to the 18-bp probe. EMSAs were performed with in vitro-made SOX9 (lanes 1 and 2), RCS cell nuclear extracts (lanes 3 and 4), and BALB/3T3 cell nuclear extracts (lanes 5 and 6). The presence (+) or absence (−) of antibodies to SOX9 is indicated. The thin arrow indicates the SOX9-DNA complex; the thick arrow indicates the supershifted SOX9-DNA complex. 18 wt, wild-type 18-bp enhancer probe. (C) Determination of the binding site of SOX9 in the *Col2a1* 18-bp enhancer. The upper strand of the wild-type 18-bp enhancer probe is shown in boldface, and flanking nucleotides are designated by lighter-face characters. It is aligned with the upper strands of mutant probes, for which only mutated nucleotides are indicated; dots indicate wild-type nucleotides. The binding site of SOX9 is boxed. Labeled probes were adjusted to identical specific activities. EMSAs were carried out with in vitro-synthesized SOX9.

The 5′ part of the 48-bp enhancer contains two other imperfect binding sites for SOX proteins (CTGTGAA and CTCTGTA), each one overlapping one side of the 5′ repeat present in this element (35). However, SOX9 was unable to bind to probes containing either one of these sites (data not shown). There appears, therefore, to be only one strong binding site for SOX9 in the 48-bp enhancer element.

SOX9 bends the *Col2a1* minimal enhancer. Several HMG domain proteins, including SRY and SOX proteins, have the ability to bend DNA (5, 6, 13, 15). This property was shown to

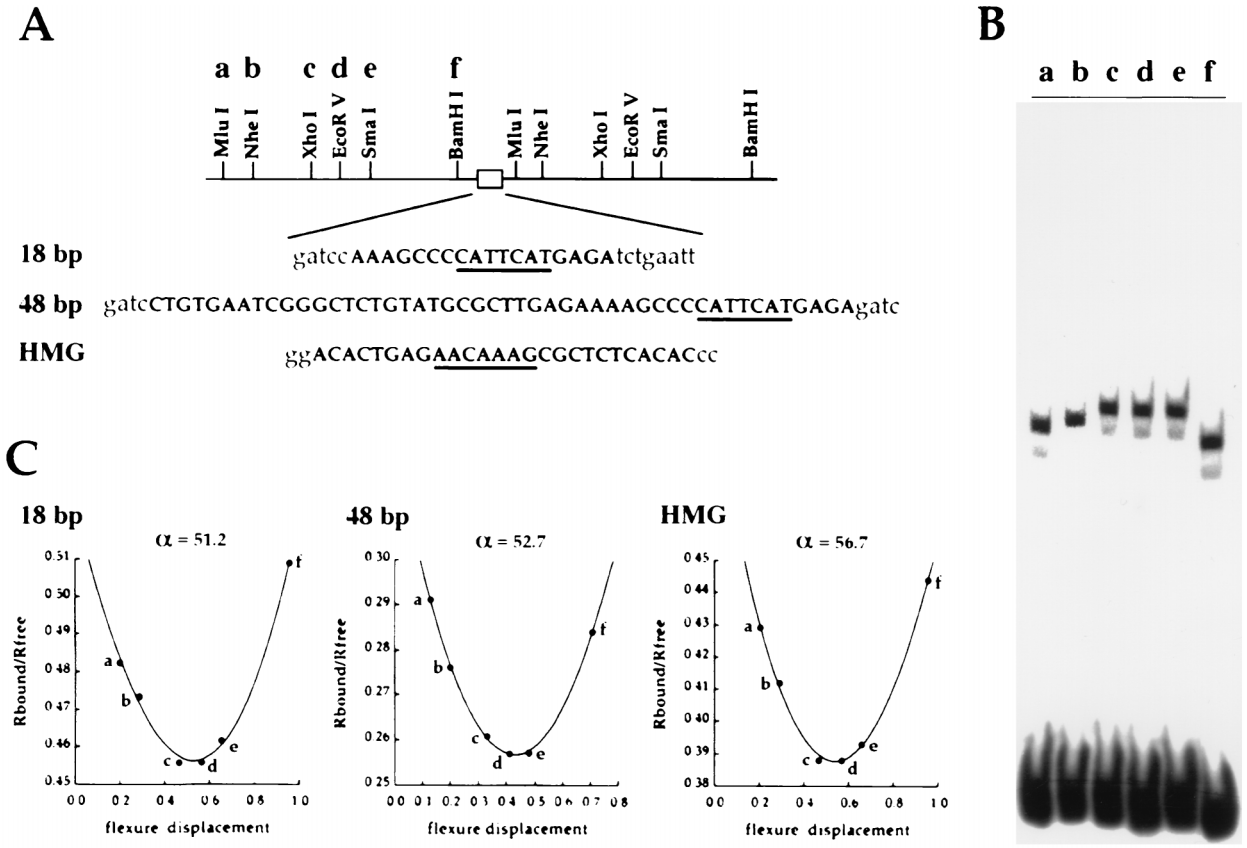

FIG. 4. Bending of the *Col2a1* enhancer by SOX9. (A) Structures of DNA probes used for the circular permutation assay. The two direct repeats present in the vector pBend2 are shown with the sites for the restriction enzymes used to generate the circularly permuted probes. These probes are designated a to f. The box indicates the site of insertion of double-stranded oligonucleotides containing the 18- or 48-bp *Col2a1* enhancer sequences or the consensus HMG domain binding site. The upper strands of these sequences are in boldface letters, with the binding site for SOX9 underlined, and linkers are in lowercase characters. (B) EMSA with in vitro-made SOX9 and the 18-bp permuted probes a to f. The top of the gel is indicated by a line. Similar results were obtained with the 48-bp and HMG probes. (C) Mapping of the center of bending to the binding site of SOX9 in the 18-bp, 48-bp, and HMG probes. The mobilities of the protein-DNA complexes (Rbound) normalized to those of the free probe (Rfree) were plotted against the flexure displacements of the probes. The flexure displacement is defined, according to Ferrari et al. (13), as the distance between the center of the SOX9 binding site and the 5′ end of the probe divided by the total length of the probe. The minimum localizes the center of bending. The estimated DNA flexure angle (α) is indicated.

allow these proteins to promote functionally important interactions between different enhancer-binding proteins (15, 16, 45). We tested the ability of SOX9 to induce DNA bending by using circularly permuted probes which contained the 18- or 48-bp *Col2a1* enhancer element or a consensus site for HMG domain proteins (Fig. 4A). SOX9 made in vitro formed complexes with these probes, the mobilities of which were clearly dependent upon the positions of the binding motifs in the probes, as shown for the 18-bp probes in Fig. 4B. The center of bending induced by SOX9, indicated by the site of flexure (Fig. 4C), mapped to the consensus binding site for SOX proteins in the HMG probe and to the binding site for SOX9 defined by EMSA in the 18- and 48-bp *Col2a1* elements. Deviation of the DNA axis induced by SOX9 was found to be similar for the HMG consensus site element and for the two *Col2a1* enhancer fragments and was estimated at 51 to 57° (Fig. 4C). These experiments demonstrate that SOX9 can induce a strong bend in DNA at its target binding site in the *Col2a1* enhancer.

SOX9 activates chondrocyte-specific *Col2a1* constructions. We asked whether forced expression of SOX9 in nonchondrogenic cells would result in activation of previously identified chondrocyte-specific *Col2a1* enhancer segments. As expected (35), a construction containing four copies of the 48-bp *Col2a1* enhancer was very active in RCS cells and inactive in BALB/3T3 fibroblasts when the cells were cotransfected with an expression vector that contained no insert (Fig. 5A). Interestingly, a strong enhancer activation occurred in BALB/3T3 cells cotransfected with the SOX9 expression plasmid. SOX9 was unable to activate a construction containing only the minimal *Col2a1* promoter (data not shown), indicating that SOX9 mediated activation through the enhancer. SOX9 also failed to activate a construction containing multiple copies of the 5′ repeat element present in the 48-bp enhancer (data not shown), which is inactive in chondrocytes (35). Constructions which had 12 copies of the minimal 18-bp enhancer (containing the 3′ repeat element) or 12 copies of a 20-bp 3′ repeat element (containing two additional nucleotides 5′ to the 18-bp sequence) were very active in RCS cells, and they became active in fibroblasts upon cotransfection with SOX9 (Fig. 5B). A mutation of nucleotides 5 and 6 in the 18-bp enhancer slightly increased enhancer activity in RCS cells and also slightly increased activation of the enhancer by SOX9 in fibroblasts. Mutations in nucleotides 1 and 2, 8 and 9, 10 and 11, or 14 and 15 abolished enhancer activity in RCS cells and also essentially abolished activation by SOX9 in fibroblasts. The same mutations in nucleotides 8 and 9, 10 and 11, or 14 and 15 also strongly reduced the binding of SOX9 to DNA (Fig. 3C), showing a good correlation between enhancer activity in chon-

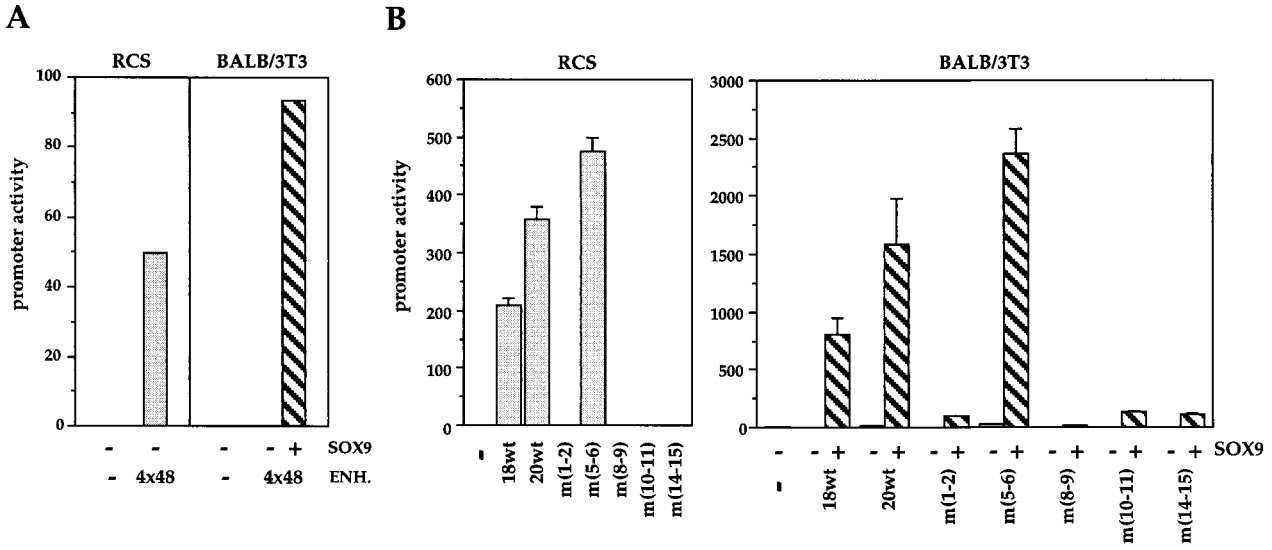

FIG. 5. Activation of the 48- and 18-bp *Col2a1* enhancer segments in fibroblasts by SOX9. (A) Cotransfection of the SOX9 plasmid with 4×48-p89Col2a1. Five micrograms of pcDNA-5′UT plasmid containing no insert (−) or encoding SOX9 (+) was mixed with the p89Col2a1 reporter plasmid containing no enhancer (ENH.) (−) or four copies of the 48-bp enhancer and with the pSV2βgal internal-control plasmid. The plasmid mixtures were transfected in RCS and BALB/3T3 cells. Promoter activities are average values for two independently transfected cultures from one representative experiment. (B) Cotransfection of the SOX9 plasmid with wild-type or mutant minimal enhancer constructions. One microgram of pcDNA-5′UT plasmid containing no insert (−) or encoding SOX9 (+) was mixed with a Col2a1-luciferase reporter plasmid and pSV2βgal. The reporter plasmid was p89Col2a1 with no enhancer element (−), 12 tandem copies of a wild-type 18- or 20-bp enhancer (18wt or 20wt), or 12 copies of a mutant 18- or 20-bp enhancer [m(1-2) to m(14-15)]. Nucleotide changes were transversion mutations. Mutations are numbered according to their positions in the 18-bp sequence printed in boldface in Fig. 3C. Plasmid mixtures were transfected in RCS cells and BALB/3T3 fibroblasts. Promoter activities are average values ± the standard deviations for three independently transfected cultures from one representative experiment.

drocytes, enhancer activation by SOX9 in fibroblasts, and binding of SOX9 to the enhancer. A mutation of nucleotides 1 and 2 did not affect the binding of SOX9 to the enhancer but abolished enhancer activation by SOX9. It is possible that this mutation abolished the binding to DNA of proteins which cooperate with SOX9 to generate enhancer activity.

Constructions with longer chondrocyte-specific *Col2a1* enhancer segments (30, 35, 40) were also tested with either one or two copies of the segments (Fig. 6). The sizes and relative locations of these segments are shown in Fig. 6A. All enhancer segments were essentially inactive in BALB/3T3 fibroblasts, but they were strongly activated by SOX9 at levels generally proportional to their activity in RCS cells (Fig. 6B). A deletion of 10 bp in the 231-bp fragment that abolishes its activity in RCS cells (35) also abolished its activation by SOX9 in fibroblasts (Fig. 6C). This 10-bp deletion included the binding site for SOX9.

In cotransfections of fibroblasts, SOX9 was also able to activate a construction which contained the *Col2a1* sequence from position −687 to +308 and in which the four copies of the 48-bp enhancer segment were placed in an intron located more than 300 bp downstream of the promoter as described by Zhou et al. (55) (data not shown). This result indicated that SOX9 was a potent activator of the *Col2a1* promoter even when its binding site was located at a distance from the transcriptional start site, which is also the case with the endogenous *Col2a1* gene.

The ability of SOX9 to activate various *Col2a1* enhancer fragments was not restricted to BALB/3T3 fibroblasts. Similar results were obtained with 10T1/2 fibroblasts, COS cells, C_2C_{12} myoblasts, and ROS 17/2.8 osteosarcoma cells (data not shown).

Altogether, these transfection experiments showed an essentially perfect correlation between the relative activities of various wild-type and mutant *Col2a1* constructions in chondrocytes and the ability of SOX9 to activate these constructions in nonchondrogenic cells.

Other members of the SOX family do not activate the *Col2a1* enhancer. Two other members of the SOX family were tested in cotransfections of COS cells (Fig. 7). The construction containing four copies of the 48-bp *Col2a1* enhancer was inactive in COS cells cotransfected with an empty expression vector, and it was activated several hundredfold by SOX9 (Fig. 7A). Sox5 was inactive, and Sox4 was only about 1/20 as active as SOX9, even though these recombinant polypeptides were synthesized in equivalent amounts (Fig. 7B). Similar results were obtained with BALB/3T3 fibroblasts (data not shown). These results demonstrated the specificity of SOX9 in the activation of the *Col2a1* chondrocyte-specific enhancer.

A truncated form of SOX9 interferes with the activity of full-length SOX9 and inhibits enhancer activity in RCS cells. A truncated SOX9 (deletion of amino acids 305 to 507), which was still able to bind efficiently to the *Col2a1* enhancer and HMG probes (Fig. 3A, lanes 2, 5, and 8), failed to induce enhancer activation in BALB/3T3 fibroblasts (Fig. 8A), confirming that the C terminus of SOX9 was important for transactivation (42). Increasing amounts of truncated SOX9 progressively inhibited the transactivation of the *Col2a1* enhancer by wild-type SOX9, probably due to competition between the two proteins for the same binding site. In RCS cells, the truncated SOX9 inhibited the activity of the *Col2a1* enhancer (Fig. 8B), suggesting that it could interfere with the activity of endogenous Sox9.

DISCUSSION

The identification of mutations in *SOX9* in patients with campomelic dysplasia and the pattern of expression of *Sox9* during embryonic development have suggested the hypothesis that SOX9 could fulfill an important function in chondrocytes.

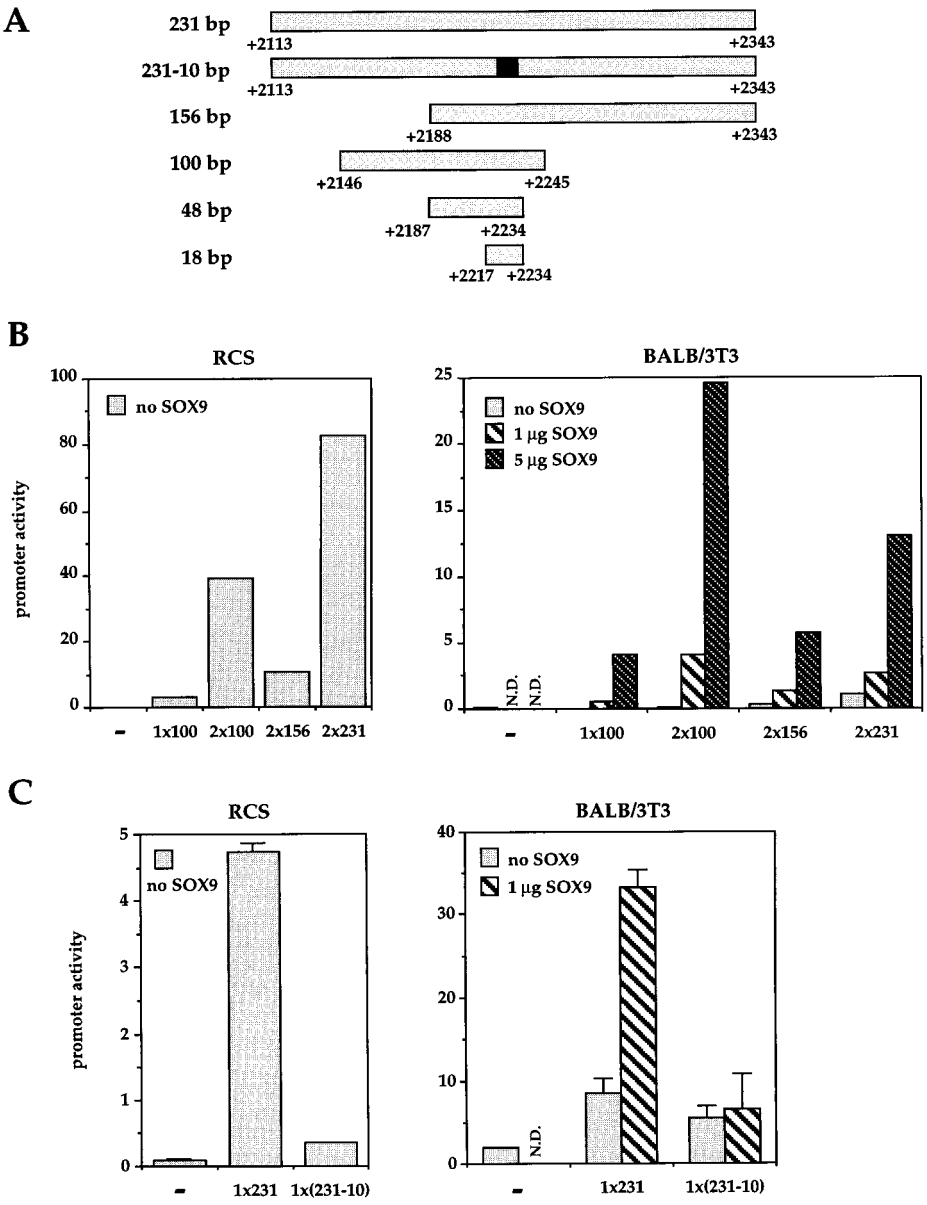

FIG. 6. Cotransfections of the SOX9 plasmid with *Col2a1* constructions containing enhancer segments of different lengths. (A) Alignment of enhancer segments. The sizes of *Col2a1* intron 1 fragments are indicated, as are the positions of the first and last nucleotides relative to the *Col2a1* transcription start site. A 10-bp deletion in the 231-bp segment (231-10 bp) is schematized by a dark box. The 48- and 18-bp enhancers are shown as references. (B) Transfection with various enhancer constructions. One microgram of pcDNA-5′UT plasmid containing no insert or encoding SOX9 was mixed with the p89Col2a1 reporter plasmid and pSV2βgal. The reporter plasmid contained no intron 1 fragment (−), one copy of the 100-bp enhancer (1×100), or two tandem copies of the 100-, 156-, or 231-bp enhancer (2×100, 2×156, and 2×231, respectively). Promoter activities in RCS cells and BALB/3T3 fibroblasts are average values for two independently transfected cultures from one representative experiment. Note that the activity of the 89-bp promoter in RCS cells (0.01 luciferase units) was significantly increased by one copy of the 100-bp enhancer (3.30 luciferase units); this enhancer construction was completely inactive in BALB/3T3 fibroblasts and significantly activated by 1 and 5 μg of SOX9 (0.59 and 4.11 luciferase units, respectively). (C) Transfection with a 231-bp enhancer construct containing a 10-bp deletion. One microgram of pcDNA-5′UT plasmid containing no insert or encoding SOX9 was mixed with pSV2βgal and p89Col2a1. The latter plasmid contained no intron 1 fragment (−) or one copy of the 231-bp or (231 − 10)-bp enhancer. Promoter activities in RCS cells and BALB/3T3 fibroblasts are average values ± the standard deviations for three independently transfected cultures from one representative experiment. The p89Col2a1 construction (no enhancer) was not tested in cotransfection with SOX9 in fibroblasts in the experiments shown in panels B and C (N.D.).

Our results first show a close correlation between the levels of expression of *Col2a1* and *Sox9* in chondrogenic cells. *Sox9* and *Col2a1* RNA levels decreased in parallel in chondrocytes during their progressive dedifferentiation in culture. In partially dedifferentiated chondrocyte cultures, *Sox9* was abundant in cells that expressed *Col2a1* whereas cells that did not express *Col2a1* contained no or low levels of Sox9. Expression of *Col2a1* in chondrogenic cell lines was associated with levels of Sox9 RNA and protein that were considerably higher than those found in nonchondrogenic cell types and tissues.

SOX9 binds to a site in the *Col2a1* enhancer which is essential for enhancer activity in chondrocytes. This site is highly homologous to the consensus binding site defined for other SOX and HMG domain proteins, and binding of SOX9 to this

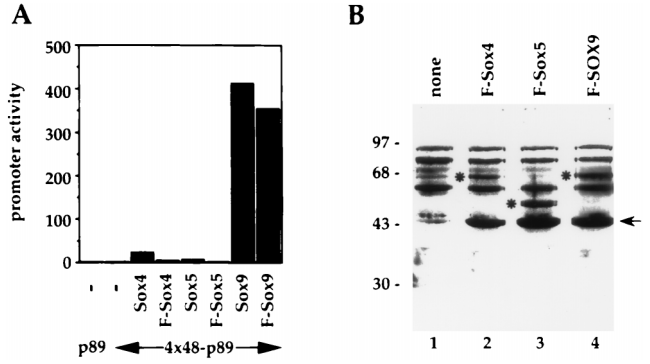

FIG. 7. Cotransfections of a *Col2a1* enhancer construction with Sox4, Sox5, and SOX9 plasmids. (A) Transfection of 4×48-p89Col2a1 with SOX plasmids. COS cells were cotransfected with pSV2βgal, together with p89Col2a1 or 4×48-p89Col2a1, and with 5 μg of pcDNA-5′UT plasmid. The latter plasmid encoded no protein (−), Sox4 without or with a FLAG epitope (F-Sox4), Sox-5, F-Sox5, SOX9, or F-SOX9. Promoter activities are average values for two independently transfected cultures from one representative experiment. (B) Western blot analysis. Lane 1, COS cells transfected with 5 μg of pcDNA-5′UT-FLAG plasmids encoding no protein (none) and 5 μg of pcDNA3.1(+) plasmid; lanes 2 to 4, cells transfected with 5 μg of pcDNA-5′UT-FLAG plasmids encoding Sox4, Sox5, or SOX9, together with 5 μg of pcDNA plasmid encoding CBF-B-FLAG (used as a control for transfection efficiency). Lysates of transfected cells were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and incubated with FLAG antibodies. FLAG-SOX proteins are shown with asterisks, and the CBF-B-FLAG protein is shown with an arrow. Protein standard M_rs are indicated on the left.

enhancer site appeared to be as efficient as binding to a consensus HMG domain site.

SOX9 was able to activate the chondrocyte-specific *Col2a1* enhancer at high levels. This was shown in transient transfections of nonchondrogenic cells with constructions harboring multiple copies of minimal enhancer segments or a single copy of larger enhancer segments, with the enhancer segments being placed either upstream of a minimal *Col2a1* promoter or downstream of a larger *Col2a1* promoter. The relative level of activation of each construction by SOX9 in fibroblasts was proportional to its activity in RCS cells. Moreover, all mutant enhancer segments (including one with a 10-bp deletion) which were inactive in chondrocytes also failed to be activated by SOX9 in fibroblasts. Hence, a perfect correlation existed between the activity of *Col2a1* enhancer constructions in chondrocytes and their ability to be activated by SOX9 in fibroblasts, strongly suggesting that SOX9 is a key activator of the *Col2a1* enhancer in chondrocytes. This enhancer is the first physiological target to be identified for SOX9.

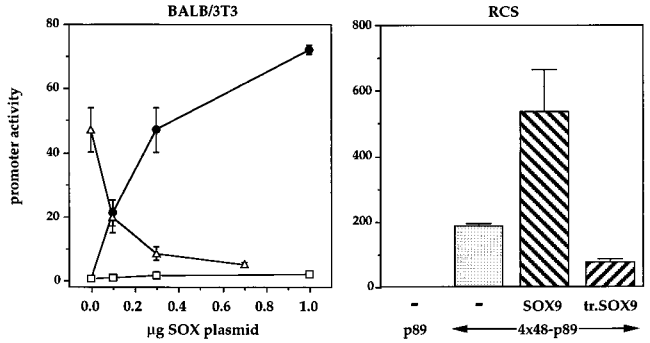

FIG. 8. Cotransfections of SOX9 and tr.SOX9 plasmids with 4×48-p89Col2a1. For transfection into BALB/3T3 fibroblasts, increasing amounts of pcDNA-5′UT plasmid encoding SOX9 (closed circles) or tr.SOX9 (open squares) were mixed with empty pcDNA-5′UT, totaling 1 μg of DNA, and 0.3 μg of SOX9-pcDNA-5′UT was mixed with empty pcDNA-5′UT and increasing amounts of tr.SOX9-pcDNA-5′UT, totaling 1 μg of DNA (open triangles); these mixtures were supplemented with 4×48-p89Col2a1 and pSV2βgal. For transfection into RCS cells, 1 μg of pcDNA-5′UT plasmid containing no insert (−) or encoding SOX9 or tr.SOX9 was mixed with p89Col2a1 or 4×48-p89Col2a1 and with pSV2βgal. Promoter activities are average values ± standard deviations for three independently transfected cultures from one representative experiment.

In contrast to the strong activation produced by SOX9, two other SOX family members, Sox4 and Sox5, were poor or ineffective activators of the *Col2a1* enhancer. In EMSA experiments, SOX9 could bind to the *Col2a1* enhancer more efficiently than Sox4 or Sox5 (data not shown). Important differences between different SOX and HMG domain proteins in binding to their cognate motifs have also been observed (5); the homologies between Sox4 and Sox9 HMG domains and between Sox5 and Sox9 HMG domains are only 59 and 40%, respectively (52). It is also possible that the transactivation domain of SOX9 is more potent than those of Sox4 and Sox5. Finally, SOX9 might interact specifically with other enhancer-binding proteins, and these interactions might potentiate the binding of SOX9 to DNA and its transactivation ability. The involvement of other proteins is suggested by the fact that a mutation in the 5′ part of the 18-bp minimal enhancer abolished activity in chondrocytes and activation by SOX9 in nonchondrogenic cells, although it did not abolish binding of SOX9 to the enhancer. This mutation might therefore abolish the binding of such putative interacting proteins. Since expression of SOX9 was sufficient by itself to activate the *Col2a1* enhancer at high levels in all nonchondrogenic cells that we tested, we postulate that these putative interacting proteins may be ubiquitous.

We have demonstrated that SOX9, like other SOX and HMG domain proteins, is able to bend DNA upon binding to a consensus HMG domain binding site and that a similar bending of DNA occurred upon binding of SOX9 to its site in the *Col2a1* enhancer. Bending of DNA by SOX9 might be crucial to facilitate interactions between other enhancer-binding proteins and between an enhancer complex and the distantly located basal transcription machinery; in *COL2A1*, the distance between the chondrocyte-specific enhancer and the promoter is over 2 kb.

In order to test whether chondrocytes express other SOX proteins that may play the same role as SOX9 in the activation of the *COL2A1* enhancer, we performed PCR assays with cDNA from primary chondrocytes and degenerate oligonucleotide primers corresponding to highly conserved regions in the HMG boxes of SOX proteins (54). In addition to cDNA for *Sox9*, sequences corresponding to *Sox5*, *Sox6*, and *Sox8* were amplified, but Northern blot analysis indicated that these genes were expressed at extremely low levels in chondrocytes (1a). Hence, the function of SOX9 in the activation of the *COL2A1* enhancer in chondrocytes does not seem to be shared with another family member.

Campomelic dysplasia is believed to be caused by haploinsufficiency of the SOX9 gene since mutations occur in only one allele. Based on the heterozygous nature of the disease and on the high levels of *SOX9* in chondrocytes compared to other cell types, including 10T1/2 and C_2C_{12} cells, we speculate that a threshold level of SOX9 is required to activate the *COL2A1* gene in chondrocytes. In campomelic dysplasia, a significant decrease in production of functional SOX9 would result in a pronounced decrease in the expression of *COL2A1*, leading to major skeletal anomalies. Several mutations detected in subjects with campomelic dysplasia result in production of truncated proteins with an intact HMG domain. These proteins have the ability to bind to DNA but lack the C-terminal tran-

scriptional activation domain. We have shown that coexpression of a truncated SOX9 protein inhibited transactivation of a *Col2a1* enhancer construction by a fully active SOX9 protein in fibroblasts and also inhibited the activity of the *Col2a1* enhancer in RCS cells. This result raised the possibility that mutations in SOX9 that cause truncation of the protein or inactivate the transactivation domain could cause campomelic dysplasia not only as a result of haploinsufficiency but also because the mutant protein would interfere with wild-type SOX9.

In previous experiments we found that the 18-bp *Col2a1* enhancer contained a binding site for POU domain proteins (35). This binding site overlaps with the SOX9 binding site. One POU domain protein which was found to bind to the *Col2a1* enhancer by EMSA with nuclear extracts of primary chondrocytes and RCS cells, but not with extracts of a series of other cell lines, was identified as Brain-1 (32a). These EMSA experiments were performed in the presence of poly(dI-dC), which prevented binding of SOX9. In situ hybridization of mouse embryos showed that in addition to its expression in neural tissues and kidney (22), *Brain-1* RNA was selectively expressed in all chondrocytes from their earliest stages of differentiation. A second POU domain protein found in RCS cells but not in other cells was identified as Brain-2. In cotransfection experiments, Brain-1 and Brain-2 were unable to activate the *Col2a1* enhancer in fibroblasts. No evidence was found that Brain-1 and Brain-2 interact with SOX9 or stabilize binding to DNA. Unlike several HMG domain proteins, which have the ability to cooperate with specific POU domain proteins (36, 54, 56), Brain-1 and Brain-2 were unable to increase the activation of the *Col2a1* enhancer by SOX9. In contrast, in cotransfection experiments in fibroblasts, expression of these POU domain proteins resulted in inhibition of SOX9 enhancer activation, possibly as a result of competition for the same binding site (data not shown). Hence, the significance of the selective expression of *Brain-1* in chondrocytes is presently not understood.

High levels of *SOX9* expression have also been found in Sertoli cells during testis development (9, 26), suggesting that SOX9 could play a role in the differentiation of Sertoli cells. This would account for the high frequency of sex reversal in patients with campomelic dysplasia. Hence, it is likely that in addition to SOX9, other specific transcription factors are needed to control the differentiation of chondrocytes and Sertoli cells.

In conclusion, our results provide evidence that the chondrocyte-specific enhancer of the *Col2a1* gene is a direct target for Sox9. This strongly suggests that *SOX9* plays a major role in the activation of *COL2A1* in chondrocytes, a major component of the differentiation program of these cells. Further studies will be necessary to identify other target genes for SOX9 in chondrocytes and to identify other transcription factors which likely contribute along with SOX9 to activation of one or several steps in the pathway of chondrocyte differentiation.

ACKNOWLEDGMENTS

We thank Alan Ashworth for Sox5 expression plasmids, Hans Clevers for Sox4 full-length cDNA, James H. Kimura for RCS cells, William T. Butler for ROS17/2.8 cells, Bjorn J. Olsen for MC615 cells, Sankar Adhya for the pBend2 plasmid, and Richard Mayne for type II collagen antibody. We are grateful to Sandra Mc Kinney and Ping Li for expert technical assistance. We thank Weimin Bi for advice with lipofectamine-based transfection method and Guang Zhou for cloning of SOX9 cDNA in pCDMA. We also thank Richard R. Behringer, William H. Klein, and Alan J. Schafer for critical review of the manuscript.

This work was funded by NIH grants AR40335 and AR42909 (to B.D.C.). V.L. was the recipient of a postdoctoral fellowship and an Arthritis Investigator Award from the Arthritis Foundation. DNA sequencing was performed by The University of Texas M. D. Anderson Cancer Center Core Sequencing Facility, which is supported by NCI grant CA16672.

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