Three High Mobility Group-like Sequences within a 48-Base Pair Enhancer of the Col2a1 Gene Are Required for Cartilage-specific Expression in Vivo*

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To understand the molecular mechanisms by which mesenchymal cells differentiate into chondrocytes, we have used the gene for an early and abundant marker of chondrocytes, the mouse pro-alpha(II) collagen gene (Col2a1), to delineate a minimal sequence needed for chondrocyte-specific expression and to identify the DNA-binding proteins that mediate its activity. We show here that a 48-base pair (bp) Col2a1 intron 1 sequence specifically targets the activity of a heterologous promoter to chondrocytes in transgenic mice. Mutagenesis studies of this 48-bp element identified three separate sites (sites 1–3) that were essential for its chondrocyte-specific enhancer activity in both transgenic mice and transient transfections. Mutations in sites 1 and 2 also severely inhibited the chondrocyte-specific enhancer activity of a 486-bp Col2a1 intron 1 sequence in vivo. SOX9, an SRY-related high mobility group (HMG) domain transcription factor, was previously shown to bind site 3, to bend the 48-bp DNA at this site, and to strongly activate this 48-bp enhancer as well as larger Col2a1 enhancer elements. All three sites correspond to imperfect binding sites for HMG domain proteins and appear to be involved in the formation of a large chondrocyte-specific complex between the 48-bp element, Sox9, and other protein(s). Indeed, mutations in each of the three HMG-like sites of the 48-bp element, which abolished chondrocyte-specific expression of reporter genes in transgenic mice and in transiently transfected cells, inhibited formation of this complex. Overall, our results suggest a model whereby both Sox9 and these other proteins bind to several HMG-like sites in the Col2a1 gene to cooperatively control its expression in cartilage.

Acquisition of the chondrocytic phenotype occurs along a major pathway of differentiation of mesenchymal cells (1, 2). With the goal of identifying transcription factors that control chondrocyte-specific gene expression, we used the gene for collagen type II (Col2a1), an early and abundant marker of chondrocytes (3–5), to delineate minimal sequences in this gene that control chondrocyte-specific expression in transgenic mice. Elucidation of the transcriptional mechanisms that control the chondrocyte-specific expression of the Col2a1 gene should provide important insights into the molecular specifications of chondrocytes.

We previously identified a 48-bp element in intron 1 of the mouse Col2a1 gene that, when present as four tandem copies, conferred chondrocyte-specific expression both in transgenic mice and in transient expression experiments in tissue culture cells (6). A multimerized 18-bp element located at the 3′ end of the 48-bp sequence also acted as a powerful chondrocyte-specific enhancer in transient transfection assays of rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not of fibroblasts (6).

SOX9 is a member of a family of transcription factors with a DNA-binding domain that shows more than 50% similarity with the high mobility group HMG DNA-binding domain of SRY, the testis-determining factor in mammals (7–12). Recently, heterozygous mutations in human SOX9 have been identified as a cause of campomelic dysplasia (CD), a severe dwarfism syndrome in which essentially all skeletal elements derived from cartilages are affected (13–17). A large proportion of genotypically male (XY) CD patients carrying mutations in SOX9 also show sex reversal. In situ hybridization during mouse embryogenesis showed that Sox9 is expressed in all chondroprogenitor cells; Sox9 expression generally parallels that of Col2a1 even in some non-chondrocytic cells and increases together with that of Col2a1 when frank chondrocyte differentiation takes place (5, 18, 19). The expression of Sox9 in gonadal ridges and later in the Sertoli cells of the testis presumably accounts for the sex reversal in CD patients (20, 21). Overall, the abnormal skeletal manifestations of CD patients and the pattern of expression of Sox9 during embryonic development suggest that Sox9 plays an important role in the pathway of chondrocyte differentiation.

Recent experiments showed that the 48-bp Col2a1 element that confers chondrocyte specificity in transgenic mice is a direct target for Sox9. Indeed, Sox9 was able to bind to a sequence in this element that is essential for chondrocyte-specific enhancer activity, and SOX9 activated this element in cotransfection experiments of nonchondrocytic cells (22). In addition, ectopic expression of SOX9 in transgenic mouse embryos

1 The abbreviations used are: Col2a1, pro-alpha(II) collagen gene; CD, campomelic dysplasia; CSEP, chondrocyte-specific enhancer-binding protein; dpc, days postcoitum; βgeo, a fusion protein with E. coli β-galactosidase and neomycin resistance activities; bp, base pair(s); HMG, high mobility group; RCS, rat chondrosarcoma cells; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosides; EMSA, electrophoretic mobility shift assays; PAGE, polyacrylamide gel electrophoresis.
bryos resulted in the activation of the endogenous Col2a1 gene in some but not all areas of ectopic SOX9 expression (23).

Although four tandem copies of the 48-bp Col2a1 sequence and 12 tandem copies of an 18-bp element within this 48-bp sequence both acted as strong chondrocyte-specific enhancers in transient expression experiments, 12 tandem copies of the 18-bp element showed much weaker activity in cartilages of the transgenic mice than did the four tandem copies of the 48-bp enhancer (6). Expression of the reporter gene in embryos harboring the multimerized 18-bp construct was also detected at low levels in skin and brain (6). Therefore, to confer high level chondrocyte-specific reporter gene expression in vivo, the entire 48-bp Col2a1 intronic fragment appeared to be needed. Although the 18-bp enhancer sequence included the SOX9-binding site of the 48-bp Col2a1 enhancer and was a strong target for SOX9 in transfection experiments (22), the above-mentioned transgenic mice results were consistent with the hypothesis that, to confer chondrocyte specificity in vivo, proteins other than Sox9 might be needed that interact with the 48-bp but not with the 18-bp enhancer sequence. The expression of Sox9 at high levels in Sertoli cells also favors the hypothesis that additional proteins are needed to differentiate the phenotype of chondrocytes from that of Sertoli cells (20, 21). Hence, the purpose of the present study was to further identify sequences in the Col2a1 48-bp enhancer essential for its chondrocyte-specific activity and to determine whether chondrocyte-specific nuclear proteins bound to these sequences. Our results indicate that the 48-bp sequence in Col2a1 contains multiple cis-acting elements essential for chondrocyte-specific expression in vivo and that chondrocytes contain specific nuclear protein(s) in addition to SOX9 that bind to these cis-acting elements and might therefore be involved in chondrocyte-specific enhancer activity.

MATERIALS AND METHODS

Cell Cultures—All cell types were obtained as described previously and cultured under standard conditions (6, 24).

DNA Constructs—To generate \(8 \times 48\text{p-globlacZ}\), eight tandem copies of the 48-bp enhancer Col2a1 element were cloned upstream of a minimal human \(\beta\)-globin promoter (\(-44\) to \(+28\)) in the reporter plasmid p lacF, as described previously for other constructs (25).

The 48-bp mutant intron 1 fragments (see Fig. 4A) were synthesized as double-stranded oligonucleotides containing BamH I- and BglII-cleaved sites at the 5‘ and 3‘ ends, respectively. These oligonucleotides were cloned and multimerized in tandem as described previously for the wild-type 48-bp construct (6). Tetramers were cloned in either the p309Col2a1-\(\beta\)-geo vector or the p89Col2a1-luc vector as described previously (6). The p309Col2a1-\(\beta\)-geo vector contained a 309-bp Col2a1 promoter and the SA-\(\beta\)-geo-bpa cassette (25). The mutant 468-bp Col2a1 intron 1 fragment (\(+1878\) to \(+2345\)) was generated by polymerase chain reaction and cloned in the p309Col2a1-\(\beta\)-geo vector as described for the wild-type element (25). The sequences of all DNA fragments generated either with oligonucleotides or by polymerase chain reaction were verified by DNA sequencing.

Transient Expression Experiments—DNA transfections were performed by the modified DNA-calcium phosphate coprecipitation method (26). Monolayers of RCS cells pre-established in 20-cm² dishes were cotransfected with 7.5 \(\mu\)g of luciferase reporter plasmids and 2.5 \(\mu\)g of pSV2\(\beta\)-gal plasmid used as an internal control for transfection efficiency. Cell extracts were prepared 40–48 h after the start of transfection, and luciferase and \(\beta\)-galactosidase activities were assayed as described (6).

Generation and Characterization of Transgenic Mice—Transgenic mice were generated as described (25). Transgenic founder embryos were sacrificed at 14.5 days postcoitum (dpc). Southern blot analysis, staining with X-gal, and \(\beta\)-galactosidase histochemical assay were performed as described (6).

Synthesis of SOX9 in Vitro and Preparation of Nuclear Extracts—SOX9 protein was synthesized by in vitro transcription-translation from a previously described SOX9-pcDNA-5′-UT expression vector (22) using the Single-tube Protein System 2 from Novagen, Inc. (Madison, WI). Nuclear extracts from all cell types were prepared as described previously (6) in buffers containing 10 \(\mu\)g/ml leupeptin and pepstatin.

Electrophoretic Mobility Shift Assays (EMSAs)—The wild-type and mutant 48-bp Col2a1 double-stranded oligonucleotides were prepared as described above under “DNA Constructs.” Bemelin and distamycin were purchased from Sigma. The OCT and HMG probes were prepared as described previously (6, 22). All probes were end-labeled with \([\alpha-32P]d\)GTP or \([\alpha-32P]d\)CTP using the Klenow fragment. Protein-DNA binding reactions were carried out as described previously (22). Assays with nuclear extracts were performed with 10 \(\mu\)g of protein and 2 \(\mu\)g of poly(dG-DC)poly(dG-DC). SOX9 synthesized in vitro was assayed in the presence of 0.1 \(\mu\)g of poly(dG-DCC)poly(dG-DCC). Supershift experiments were performed with purified SOX9 antibodies as described previously (22).

Elution of CSEP from Electrophoresis Gels—RCS cell nuclear extracts were partially purified by passage through a DNA affinity column containing an R2 oligonucleotide containing an \(\beta\) globin promoter. The staining pattern is very similar to that for the stage-matched transgenic embryo carrying p309\(4 \times 48\text{Col2a1}\) shown in Fig. 6B. C and D, sagittal sections of the embryo shown in B: me, Meckel’s cartilage.

RESULTS

Chondrocyte-specific Targeting by a 48-bp Col2a1 Element in Transgenic Mice—We showed previously that a 48-bp sequence of intron 1 in the Col2a1 gene together with a 309-bp Col2a1 promoter conferred strict chondrocyte-specific expression in transgenic mice (6). To investigate further whether the 48-bp Col2a1 sequence by itself contained all the cis-acting elements needed for chondrocyte-specific enhancer activity in intact mouse embryos, we generated a transgene \(i(8 \times 48\text{p-globlacZ})\) in which eight tandem repeats of the 48-bp enhancer fragment were cloned upstream of a minimal human \(\beta\)-globin promoter (Fig. 1A). Two of the five transgenic 14.5-dpc founder embryos harboring \(i(8 \times 48\text{p-globlacZ})\) stained positive for X-gal, a chro-
Functional Analysis of a 48-Base Pair Col2a1 Enhancer

Identification of chondrocyte-specific protein(s) binding to the 48-bp Col2a1 element. EMSA was performed using the 48-bp probe and nuclear extracts from the following cell types: primary mouse rib chondrocytes (Pr.Ch.); MC615 mouse immortalized chondrocytes; RCS cells; 10T1/2 mouse embryo fibroblasts; ROS 17/2.8 rat osteosarcoma cells; C2C12 and C2C7 mouse skeletal myoblasts; subline 714 of BALB/3T3 mouse embryo fibroblasts; EL4 mouse lymphoma T-type cells; Raji human lymphoblast-like cells; S194 mouse myeloma cells; NMuLi mouse normal liver cells; RAG mouse renal adenocarcinoma cells; and Hep3B human hepatocellular carcinoma cells. Arrowhead, chondrocyte-specific CSEP-48 bp DNA complex.

Fig. 2. Identification of chondrocyte-specific protein(s) binding to the 48-bp Col2a1 element. EMSA was performed using the 48-bp probe and nuclear extracts from the following cell types: primary mouse rib chondrocytes (Pr.Ch.); MC615 mouse immortalized chondrocytes; RCS cells; 10T1/2 mouse embryo fibroblasts; ROS 17/2.8 rat osteosarcoma cells; C2C12 and C2C7 mouse skeletal myoblasts; subline 714 of BALB/3T3 mouse embryo fibroblasts; EL4 mouse lymphoma T-type cells; Raji human lymphoblast-like cells; S194 mouse myeloma cells; NMuLi mouse normal liver cells; RAG mouse renal adenocarcinoma cells; and Hep3B human hepatocellular carcinoma cells. Arrowhead, chondrocyte-specific CSEP-48 bp DNA complex.

mogenic substrate for β-galactosidase, whereas the other three did not. Moreover, the X-gal-positive embryos harboring i(8 × 48)pglb lacZ exhibited cartilage-specific staining similar to that seen at the same developmental stage (Fig. 1B) in embryos harboring transgene p309/i(4 × 48)Col2a1, in which the same 48-bp fragment was driving a 309-bp Col2a1 promoter (Fig. 6B). X-Gal staining was observed in the cartilages of the head, scalpula, vertebrae, ribs, limbs, and shoulder and pelvic girdles. Histological analysis showed that X-gal staining was present in chondrocytes only; no promiscuous X-gal staining was detected in any nonchondrogenic tissues (examples are shown in Fig. 1, C and D). Hence, these experiments demonstrated that in transgenic embryos the sequence of the 48-bp Col2a1 intron 1 element contained the essential elements required to target the activity of a minimal heterologous promoter specifically to chondrocytes.

Formation of a Chondrocyte-specific Complex with the 48-Base Pair Col2a1 Enhancer Element—In previous EMSA experiments, an 18-bp Col2a1 enhancer probe was used to identify Sox9 and other chondrocyte-enriched proteins in nuclear extracts of primary chondrocytes and RCS cells (6, 22). Since the 48-bp Col2a1 element, which includes the 18-bp sequence, confers a much stricter chondrocyte specificity in transgenic embryos than does the 18-bp element, the 48-bp Col2a1 element was used as a probe in EMSA experiments to (a) identify in chondrocytes unique nuclear factors that would specifically bind to the 48-bp enhancer and (b) locate the DNA-binding sites for these factors.

Formation of a major complex with the 48-bp enhancer probe was observed in reactions using extracts from primary chondrocytes and from two chondrocytic cell lines (MC615 and RCS cells) (Fig. 2). This complex was absent in reactions with extracts from fibroblastic cell lines (10T1/2 and Balb/3T3) and all other nonchondrogenic cell lines tested. The protein(s) forming the major complex was tentatively named CSEP for chondrocyte-specific enhancer-binding proteins.

Interestingly, SOX9 synthesized in vitro formed a major complex with the 48-bp probe (Fig. 3A, lane 1) whose mobility was similar to that of the complex formed between the 18-bp probe and Sox9 present in crude chondrocyte extracts (22), yet no complex with a similar mobility could be seen when the same chondrocyte extracts were incubated with the 48-bp probe under similar EMSA conditions (Fig. 3A, lane 2). The CSEP-DNA complex migrated more slowly than the major complex formed between SOX9 made in vitro and the 48-bp probe; a second complex that often formed between SOX9 synthesized in vitro and the 48-bp probe migrated at the trailing edge of the CSEP-DNA complex. To test whether Sox9 was part of the CSEP complex, we performed supershift experiments with Sox9 antibodies (22). These antibodies were able to completely supershift the major complexes and the second complexes formed between the 48-bp probe and SOX9 made in vitro (data not shown) as well as the complex between the 18-bp probe and Sox9 present in RCS cell nuclear extracts (22). When chondrocyte nuclear extracts were incubated with the 48-bp probe, we observed the formation of a supershift complex with SOX9 antibodies, but despite extensive efforts, the CSEP-DNA complex was always either unaffected or only slightly weaker than in control reactions without antibodies (Fig. 3A, lanes 3 and 4). We concluded that Sox9 could be part of the CSEP complex and hypothesized that CSEP might contain one or more other proteins.

Efficient formation of a complex between CSEP and the 48-bp probe was seen in EMSA in which poly(dI-dC) was used as nonspecific competitor but not when poly(dA-dT) or poly(dI-dC) was used (data not shown). Since poly(dI-dC) mimics A-T pairs in the minor but not the major groove of DNA (27, 28), these data suggested that CSEP might interact with A-T pairs in the minor groove of the 48-bp probe. In support of this possibility, two A-T pair-selective minor groove DNA ligands distamycin or berenil (diminazene acetate) (29, 30) inhibited CSEP binding to the 48-bp probe at concentrations as low as 1 μM (Fig. 3B). In control experiments (data not shown), concentrations of 1 μM distamycin and berenil had no effect on the binding to the 18-bp Col2a1 enhancer element of three POU-domain proteins (Oct-1, Brain-1, and Brain-2) previously identified in RCS cell nuclear extracts (6, 22) and known to bind DNA mainly in the major groove (31).

HMG domain proteins are known to contact A-T pairs in the minor groove of the DNA helix, and their binding to DNA can be blocked by distamycin and berenil (29, 30). This suggested, therefore, that the CSEP complex could involve HMG-like proteins. To test this hypothesis, we performed EMSA competition experiments between the 48-bp enhancer probe and an oligonucleotide containing a consensus heptamer-binding site for HMG domain proteins (HMG oligonucleotides) (22, 32). The HMG oligonucleotide competed about 10 times more efficiently than did the 48-bp element for the formation of a complex with CSEP (Fig. 3C). In a control experiment, a 300-fold excess of a consensus octamer-binding site for POU domain proteins (6) failed to compete with the labeled 48-bp probe for the formation of a complex with CSEP.

To test whether other HMG-like proteins, in addition to Sox9, could be involved in CSEP-48 bp probe complex formation, we performed direct binding assays with the HMG probe (Fig. 3D, lanes 2–11). Several complexes were formed by incubating the HMG probe with nuclear extracts from primary chondrocytes. Interestingly, complex 1 had a mobility similar
to that of the CSEP-48-bp probe complex and as the CSEP-48-bp complex was formed with extracts from primary chondrocytes and the chondrocytic RCS and MC615 cells but not with extracts from the fibroblastic 10T1/2 and Balb/3T3 cells. This complex was not affected by addition of SOX9 antibodies. Complementary experiments indicated that 100-fold excess amount of the 48-bp probe could compete successfully with the HMG probe for the formation of complex 1 (data not shown). Complex 2 appeared to correspond to Sox9, as demonstrated by supershifting with specific antibodies and by formation of this complex with extracts from cells that express Sox9, i.e. chondrocytic cells (lanes 2–7) and 10T1/2 cells (lanes 8 and 9). Hence, these data suggested the hypothesis that CSEP-48-bp complex might involve a protein that has the ability to bind to an HMG oligonucleotide independently of Sox9.

To test this hypothesis further, proteins were eluted from gel slices after SDS-PAGE of RCS cell nuclear extracts. In EMSA experiments, eluates containing proteins with an apparent M_r of 75,000–95,000 formed a complex with the same mobility as the CSEP-48-bp complex (Fig. 3E); formation of this complex was inhibited by poly(dI-dC) but not poly(dG-dC). These 75–95-kDa proteins also formed a complex with the HMG probe that had the same mobility as complex 1 in Fig. 3D (data not shown). The complex formed between these proteins and the 48-bp DNA could not be supershifted with SOX9 antibodies, a result in agreement with our previous Western blot experiments in which Sox9 was detected as a unique protein(s) with an apparent M_r of 75,000–95,000.

SDS-PAGE was performed with partially purified RCS cell nuclear extracts, protein was eluted from 16 gel slices schematically shown at left (the molecular weight of protein standards is indicated), renatured, and tested in EMSA with the 48-bp probe. Crude nuclear extracts from RCS cells were used as the EMSA standard.
site 2 (CTTTTCT) (Fig. 4A, wild-type sequence). To determine whether CSEP binds to these HMG-like sites, oligonucleotide probes with mutations in these sites were generated (Fig. 4A, MA1 to MA8) and tested in EMSAs. Formation of the CSEP-DNA complex was decreased when mutant probes which disrupted any one of these sites (MA1, MA2, MA4, MA5, and MA6) were used (Fig. 4B). When MA8 (which harbored mutations in all three sites) was used, formation of the CSEP-DNA complex was essentially abolished. When MA3 (which retained five of the seven nucleotides of the consensus HMG site in site 2) was used, formation of the CSEP-DNA complex was decreased to a lesser degree than when MA2, MA4, or MA5 (which retained only three of seven nucleotides of the HMG consensus site in site 2) was used. Interestingly, formation of the CSEP-DNA complex was strongly enhanced by using mutant MA7, in which site 3 corresponded to a consensus HMG site. Hence, these experiments indicated that binding of CSEP to the 48-bp sequence involved all three HMG-like sites.

Our previous experiments showed that SOX9 binds to site 3 and produces a strong bend in the 48-bp DNA at this site (22). Therefore we asked whether the different mutations in the 48-bp element affected binding of SOX9 (Fig. 4C). As expected, mutation MA6, which disrupted site 3, abolished binding of SOX9 to the 48-bp probe; mutation MA7, which generated a perfect HMG site at site 3 and increased formation of the CSEP-DNA complex, had no significant effect on SOX9 binding to the 48-bp probe. Mutations in sites 1 and 2 (MA1 and MA4), which decreased formation of the CSEP-DNA complex, had little or no effect on SOX9 binding to the 48-bp probe (Fig. 4C). Hence, these results confirmed that SOX9 binds site 3 and indicated that mutations affected differently binding of CSEP and SOX9 to the 48-bp probe, suggesting again that CSEP contains components other than Sox9 or in addition to Sox9.

Abolition of Chondrocyte-specific Enhancer Activity by Mutations in Three HMG-like Sites—To test the effect of mutations in the HMG-like sites of the Col2a1 48-bp element on enhancer activity in RCS cells, four tandem repeats of mutant 48-bp elements were cloned into the luciferase vector (Fig. 5). Mutations that strongly decreased the binding of CSEP, i.e., MA1, MA4, and MA6, abolished enhancer activity in RCS cells (Fig. 5). Mutation MA3, which had a much weaker effect on formation of the CSEP-DNA complex in EMSA, had little effect on enhancer activity in RCS cells. Mutation MA7, which increased formation of the CSEP-DNA complex in EMSA, had no significant effect on enhancer activity. Hence, there was a good correlation between formation of the CSEP-48-bp Col2a1 DNA
complex and enhancer activity in RCS cells as assayed in transient transfection experiments.

To investigate the in vivo effect of mutations in the three HMG-like sites of the 48-bp enhancer that affected formation of the CSEP DNA complex, we generated transgenic mice harboring four tandem copies of the 48-bp mutant elements cloned as shown in Fig. 6A (25). As shown previously (6), the wild-type 48-bp element was sufficient to confer chondrocyte-specific expression in transgenic mice (Fig. 6B). However, mutations in any one of the three HMG-like sites 1, 2, and 3 (i.e. MA1, MA4, and MA6) that decreased the formation of the CSEP-DNA complex in EMSA and abolished enhancer activity in transiently transfected RCS cells resulted in loss of chondrocyte-specific transgene expression in vivo. In 14.5-dpc embryos harboring mutant 48-bp elements, promiscuous X-gal staining was detected in various nonchondrogenic tissues such as brain, tongue, tendons, dermis, and spinal cord (Fig. 6, C–E, and Table I). This promiscuous expression pattern varied among individual embryos. Histological analysis confirmed that X-gal staining occurred in these nonchondrogenic tissues (data not shown). In some embryos harboring either mutant MA1 (site 1 mutated) or mutant MA4 (site 2 mutated), occasional weak X-gal staining was also observed in some rib or limb chondrocytes. In contrast, in embryos harboring mutant MA7 (site 3 mutated), which increased the formation of the CSEP-DNA complex and had no significant effect on enhancer activity in transient transfection experiments of RCS cells, β-galactosidase activity was high and specifically targeted to chondrocytes (Fig. 6, F–H). In conclusion, mutations in three different sites of the 48-bp element that resulted in decreased formation of the CSEP-48-bp DNA complex abolished or greatly inhibited chondrocyte-specific expression in transgenic mouse embryos (Table II).

We previously showed that a single copy of a 468-bp mouse Col2a1 intron 1 element (+1878 to +2345), which includes the 48-bp enhancer, conferred pronounced and strict chondrocyte-specific expression in all cartilages of transgenic embryos (25) (Fig. 7, B and D). Hence, to determine whether the HMG-like sites 1 and 2 were also essential for the activity of this larger intron 1 fragment, we generated mouse embryos harboring a construct containing a single copy of the 468-bp fragment with both of the mutations present in MA1 and MA4 (Fig. 7A). Unlike embryos harboring the wild-type 468-bp construct, six transgenic mouse embryos harboring this mutant 468-bp construct showed X-gal staining but none that was cartilage-specific. Promiscuous X-gal staining occurred in eyes, ears, vibrissae, interdigital areas, and skin (Fig. 7C and data not shown). Histological analysis also showed X-gal staining in dermis and tendons (Fig. 7E). There was weak, limited X-gal staining in a subset of chondrocytes of some cartilages in some of these mice. Together these results indicated that the HMG-like sites 1 or 2 are essential for achieving the in vivo high level chondrocyte-specific activity of the single copy 468-bp Col2a1 intron 1 fragment.

**DISCUSSION**

In 14.5-dpc transgenic mouse embryos, four tandem repeats of the 48-bp enhancer in combination with a 309-bp Col2a1 promoter targeted the expression of a β-galactosidase reporter gene to chondrocytes specifically (6). Transgenic mouse embryos harboring a 309-bp Col2a1 promoter that lacked intron 1 enhancer sequences showed no β-galactosidase expression in chondrocytes (25). Now we have shown that when the Col2a1 promoter is replaced with a minimal β-globin promoter, the multimerized 48-bp intron 1 Col2a1 element is still able to target expression of the lacZ transgene specifically to chondrocytes in 14.5 dpc transgenic mouse embryos, indicating that Col2a1 promoter sequences are dispensable for chondrocyte-specific expression in intact mouse embryos.

In previous transient expression experiments, 12 tandem copies of an 18-bp sub-element of the 48-bp sequence were a more potent chondrocyte-specific enhancer than four tandem copies of the 48-bp element (6). However, in transgenic mice, the 12 tandem copies of the 18-bp sequence were much less effective in conferring a high level and strict chondrocyte specificity in vivo (6). We therefore hypothesized that additional sequences outside the 18-bp segment in the 48-bp element were needed to achieve such high level chondrocyte-specific expression in vivo. Our present results show that mutations in three different sites of the 48-bp element resulted in loss of chondrocyte-specific enhancer activity both in transgenic mouse embryos and in transient expression experiments. Two of these sites, sites 1 and 2, are located outside the 18-bp element (only the 3′ part of site 2 is present in the 18-bp sequence), which includes the previously identified Sox9-binding site (site 3) (6, 22). All three mutations disrupt potential binding sites for HMG domain proteins.

When nuclear extracts from chondrocytes were used in EMSAs with the 48-bp probe, no complex formed that had the mobility of the major complex formed between SOX9 synthesized in vitro and the 48-bp probe. However, a prominent, slower migrating complex was formed with nuclear proteins present in primary chondrocytes and chondrocytic cell lines but not in other cell lines (CSEP). Since a limited supershift of the multimerized 48-bp complex was observed with SOX9 antibodies, it is likely that Sox9 was present in the complex. However, because SOX9 antibodies supershifted only a fraction of the CSEP-48-bp DNA complex, we hypothesized that the CSEP-48-bp DNA complex also included one or more proteins distinct from Sox9.

Additional lines of evidence further support this hypothesis. First, although Sox9 is present in 10T1/2 cells, no CSEP-48-bp DNA complex was formed with nuclear extracts of these cells.
Second, after fractionation of chondrocyte nuclear extracts by SDS-PAGE followed by protein elution and renaturation, a protein or proteins with an apparent Mr of 75,000–95,000 formed a complex with the 48-bp sequence that had the same mobility and the same DNA-binding properties as the CSEP-48-bp DNA complex; this complex was not supershifted by SOX9 antibodies. As Western blot analysis revealed, SOX9 ran as a unique species with an apparent Mr of 68,000 (22). Our data also suggest that proteins present in the CSEP-48-bp complex, which are different from Sox9, are HMG-like proteins.

TABLE I
| Sequence | CSEP binding | Activity in RCS cells | Chondrocyte-specific expression in transgenic mice | SOX9 binding |
|----------|--------------|-----------------------|-----------------------------------------------|--------------|
| WT 5′-CTGTGAACTCGGGCTCTGTATGCGCTTGAGAAAAGCCCCATTCATGAGA-3′ | ++ + | + | Yes | + |
| MA1 5′-..........................ac...............................–3′ | + | – | No | + |
| MA3 5′-..........................ct...............................–3′ | ++ | + | Not tested | + |
| MA4 5′-..........................ct...............................–3′ | + | – | No | + |
| MA6 5′-..........................ct...............................–3′ | + | – | No | + |
| MA7 5′-..........................ct...............................–3′ | ++ + + | Yes | + | + |

* Only the coding strand is shown; the three HMG-like sites are underlined, and mutated nucleotides are in lowercase letters. The binding site of SOX9 is boxed.

TABLE II

Summary of in vivo and in vitro properties of mutant 48-bp Col2a1 enhancer elements

Fig. 6. Abolition of chondrocyte-specific expression in transgenic mouse embryos by mutations in the 48-bp Col2a1 enhancer element. A, schematic representation of the transgenes. Four tandem copies of wild-type (WT) or mutant 48-bp elements (MA1 to MA7, as shown in Fig. 4A) were cloned in the p309Col2a1-βgeo vector (25). The p309Col2a1-βgeo vector contains the 309-bp Col2a1 promoter, exon 1 (+1 to +237) in which the translation start sequence has been mutated from ATG to CTG, and 5′ part of intron 1 (+238 to +308). The Col2a1 enhancer elements were inserted between +308 and the SA-βgeo-bpA cassette; this cassette includes a splice acceptor (SA), the βgeo gene, which encodes a fusion protein with Escherichia coli β-galactosidase and neomycin resistance activities, and the bovine growth hormone gene polyadenylation signal (bpA). B–F, lateral views of representative whole mount transgenic founder embryos harboring four tandem copies of wild-type (B) or mutant 48-bp elements (MA1, MA4, MA6, MA7, respectively) (C–F) stained with X-gal at 14.5 dpc. G and H, sagittal sections of the embryo shown in F. ve, vertebra; di, cartilage of digit.

FIG. 6.
Abolition of chondrocyte-specific expression in transgenic mouse embryos by mutations in the 48-bp Col2a1 enhancer element. A, schematic representation of the transgenes. Four tandem copies of wild-type (WT) or mutant 48-bp elements (MA1 to MA7, as shown in Fig. 4A) were cloned in the p309Col2a1-βgeo vector (25). The p309Col2a1-βgeo vector contains the 309-bp Col2a1 promoter, exon 1 (+1 to +237) in which the translation start sequence has been mutated from ATG to CTG, and 5′ part of intron 1 (+238 to +308). The Col2a1 enhancer elements were inserted between +308 and the SA-βgeo-bpA cassette; this cassette includes a splice acceptor (SA), the βgeo gene, which encodes a fusion protein with Escherichia coli β-galactosidase and neomycin resistance activities, and the bovine growth hormone gene polyadenylation signal (bpA). B–F, lateral views of representative whole mount transgenic founder embryos harboring four tandem copies of wild-type (B) or mutant 48-bp elements (MA1, MA4, MA6, MA7, respectively) (C–F) stained with X-gal at 14.5 dpc. G and H, sagittal sections of the embryo shown in F. ve, vertebra; di, cartilage of digit.

TABLE I

| Ratios of transgenic founder embryos positive by X-gal staining versus embryos positive by genomic Southern blot analysis and expression pattern of transgenes in 14.5 dpc embryos |
|-----------------|----------------|----------------|----------------|----------------|
| WT 5′-CTGTGAACTCGGGCTCTGTATGCGCTTGAGAAAAGCCCCATTCATGAGA-3′ | ++ + | + | Yes | + |
| MA1 5′-..........................ac...............................–3′ | + | – | No | + |
| MA3 5′-..........................ct...............................–3′ | ++ | + | Not tested | + |
| MA4 5′-..........................ct...............................–3′ | + | – | No | + |
| MA6 5′-..........................ct...............................–3′ | + | – | No | + |
| MA7 5′-..........................ct...............................–3′ | ++ + + | Yes | + | + |

* Only the coding strand is shown; the three HMG-like sites are underlined, and mutated nucleotides are in lowercase letters. The binding site of SOX9 is boxed.
Indeed, a complex with a mobility and DNA-binding properties similar to those of the CSEP complex was competed by the 48-bp probe. In addition, this complex was formed with extracts of RCS cells that was decreased in the presence of two intact Sox9-binding sites in the fragment. This suggests that several HMG-like binding sites need to be occupied by transcription factors in order to activate the single copy 468-bp transgene at high levels in all cartilages in vivo.

Overall our results suggest a model in which both Sox9 and other proteins present in the CSEP-DNA complex bind to several HMG-like sites in the chondrocyte-specific Col2a1 gene to control its chondrocyte-specific expression. To identify these other proteins and study their function, the cDNAs for these proteins will need to be cloned.

In experiments reported in the accompanying article (38), we have identified two short chondrocyte-specific enhancer elements within the promoter of the mouse gene for the α2 subunit of type XI collagen (Col11a2), a gene that is expressed preferentially in chondrocytes. Like the Col2a1 48-bp enhancer both elements contain several HMG-like sites and formed a DNA-protein complex with extracts of RCS cells that was dependent on the sequence of these sites. Furthermore, these complexes had the same mobility as the CSEP-48-bp Col2a1 complex and, similarly, appeared to contain SOX9 and other proteins. Like the Col2a1 enhancer, the Col11a2 elements were also able to activate reporter genes in chondrocytes, but not in fibroblasts, and were activated by forced expression of SOX9 in non-chondrocytic cells. Both Col11a2 elements also directed transgene expression to chondrocytes in mouse embryos. On the basis of these similarities, we speculate that common mechanisms involving both Sox9 and the other proteins present in CSEP may control the chondrocyte-specific expression of the Col2a1 and Col11a2 genes and perhaps a larger genetic program of chondrocyte differentiation.

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