Chondrocyte-specific Enhancer Elements in the Col11a2 Gene Resemble the Col2a1 Tissue-specific Enhancer*

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Laura Clarke Bridgewater‡, Véronique Lefebvre§, and Benoit de Crombrugghe¶

From the Department of Molecular Genetics, the University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Type XI collagen and type II collagen are coexpressed in all cartilage, and both are essential for normal cartilage differentiation and skeletal morphogenesis. This laboratory has recently identified a 48-base pair (bp) enhancer element in the type II collagen gene Col2a1 that contains several HMG-type protein-binding sites and that can direct chondrocyte-specific expression in transient transfection and in transgenic mice. The present study has identified two short chondrocyte-specific enhancer elements within a region in the 5′ portion of the type XI collagen gene Col11a2 that has previously been shown to influence chondrocyte-specific expression in transgenic mice. These Col11a2 enhancer elements, like the Col2a1 enhancer, contain several sites with homology to the high mobility group (HMG) protein-binding consensus sequence. In electrophoretic mobility shift assays, the Col11a2 elements formed a DNA-protein complex that was dependent on the presence of the HMG-like sites. It had the same mobility as the complex formed with the Col2a1 48-bp enhancer and appeared to contain the same or similar proteins, including SOX9. The Col11a2 elements directed gene expression in transient transfections of chondrocytes but not fibroblasts, and their activity was abolished by mutation of the HMG-like sites. Ectopically expressed SOX9 activated these enhancers in non-chondrocytic cells, as it also activates the Col2a1 enhancer. Finally, the Col11a2 enhancer elements both directed transgene expression to cartilage in developing mouse embryos. Overall, our results indicate that the two Col11a2 chondrocyte-specific enhancer elements share many similarities with the Col2a1 48-bp enhancer. These similarities suggest the existence of a genetic program designed to coordinately regulate the expression of these and perhaps other genes involved in the chondrocyte differentiation pathway.

Type XI collagen, like type II collagen, is expressed primarily in cartilage and is an essential component of cartilage collagen fibrils. A mutation in the gene for its α1(XI) subunit, Col11a1, is responsible for the abnormal cartilage and skeletal defects observed in cho/cho mice (1). Likewise, mutations in the gene for its α2(XI) subunit, Col11a2, have been linked to both autosomal dominant and recessive osteochondrodysplasias in humans (2). Mutations in the type II collagen gene Col2a1 also compromise the function of cartilage, resulting in skeletal abnormalities (3–5).

Recent work in this laboratory has identified a 48-bp enhancer element within the first intron of the Col2a1 gene which, when multimerized as four tandem copies, is active in chondrocytic cells but not in fibroblasts in transient transfection experiments. In transgenic mice, this multimerized enhancer is sufficient to direct transgene expression specifically to the cartilage of developing embryos (6). The element is a direct target of binding by the transcription factor SOX9 (7).

The skeletal malformation syndrome campomelic dysplasia results from mutations in and around the SOX9 gene (8–10). The SOX9 protein contains an HMG-type DNA-binding domain and a transactivation domain that has been mapped to the C terminus (11). It is expressed abundantly in chondrocytes and in chondroprogenitor cells, and its expression closely parallels that of Col2a1 in developing mouse embryos (12–15). SOX9 can activate the 48-bp chondrocyte-specific enhancer of Col2a1 in non-chondrocytic cells in transient transfection experiments (7). Furthermore, ectopic expression of a SOX9 transgene in mice can activate expression of the endogenous Col2a1 gene in some tissues (16). Together, these data suggest that SOX9 plays an important role in chondrogenesis, perhaps by directly activating Col2a1 expression.

The accompanying paper (28) demonstrates that, in addition to the SOX9-binding site, other HMG-like sites are present in the 48-bp Col2a1 chondrocyte-specific enhancer. These sites participate in the formation of a large chondrocyte-specific protein complex that includes Sox9 and other proteins which appear to belong to the HMG domain protein family. The proteins forming this complex were tentatively designated CSEP (for chondrocyte-specific enhancer-binding proteins) (28). Mutations that inhibited binding of CSEP to the various HMG-like sites also abolished the chondrocyte-specific enhancer activity of the 48-bp element in DNA transfection experiments and in transgenic mice. This correlation between CSEP binding and enhancer activity suggests that the CSEP proteins may play a role in the tissue-specific expression of Col2a1 and hence in chondrogenesis.

Because Col11a2 has basically the same expression pattern as Col2a1, we wondered if it might also share a similar tissue-specific regulatory mechanism. The goal of the present work was to determine whether the Col11a2 gene contains a chon-
Chondrocyte-specific Col11a2 Enhancer Elements

Previous transgenic mouse experiments have demonstrated that the entire Col11a2 promoter from −742 bp to +380 bp can direct expression to essentially all cartilaginous structures, whereas a truncated promoter showed no tissue-specific activity (17). One interpretation of this result is that the region of Col11a2 eliminated in the truncation may be involved in regulating the tissue-specific expression of the gene. Examination of the sequence of Col11a2 from the −742 bp to +380 bp revealed a consensus HMG domain protein-binding site that we show to be essential for the chondrocyte-specific activity of this promoter in transgenic mice. There are also additional sites with homology to the HMG consensus, reminiscent of the promoter in transgenic mice. There are also additional sites with homology to the HMG consensus, reminiscent of the promoter in transgenic mice. We show that these sites are necessary for enhancer activity. Finally, we show that the elements are capable of directing chondrocyte-specific gene expression in transient transfection and in transgenic mice.

EXPERIMENTAL PROCEDURES

Cell Types and Culture—The rat chondrosarcoma RCS cell line was obtained as described previously (18). BALB/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD), and the 714 subline was used (19). Both cell lines were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), l-glutamine (2 mM), and 10% calf serum (Life Technologies, Inc.).

DNA Probes—The Col11a2 probes were named by designating the putative HMG domain protein-binding sites in the region of interest A, B, C, D, and E, with A being most 5′ (Fig. 1, panel A). When designing mutated probes (Fig. 1, panel B), the likelihood that mutated sites in the A/B/C and D/E probes would be recognizable to HMG domain proteins was maximized by introducing mutations to give a total of 4 mismatches with the 7-bp consensus, by introducing mutations at the invariable sites in the consensus, and by substituting G or C for A or T residues to eliminate the overall A/T-rich nature of the consensus site. Dmut is the same as Dwt but with two mutations within the HMG site, representing change of the HMG consensus sequence than the other mutant probes contain. All DNA probes were made as complementary oligonucleotides (Genosys; The Woodlands, TX) with 5′ overhangs for radiolabeling and cloning. The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before annealing.

Plasmid Constructions—The pCXIwt plasmid was constructed exactly as described by Tsumaki et al. (17) and named 742lacZ by them. Briefly, a 1,122-bp fragment between −742 and +380 of Col11a2 was amplified by PCR using a cosmid containing the entire mouse Col11a2 gene as template. (This cosmid was a kind gift of Dr. Darwin Prockop at Allegheny University of the Health Sciences, Philadelphia, PA.) The PCR product was cloned into the EcoR1XhoI polynucleotid region in the expression vector pNASSβ (CLONTECH Inc., Palo Alto, CA), which contains a full-length Escherichia coli β-galactosidase reporter gene, and a splice donor/splice acceptor and polyadenylation signal from SV40, but no promoter of its own. The pCXIwt plasmid is like pCXIwt except that it contains a 2-bp mutation (identical to that contained in probe Dmut) within the consensus HMG domain binding site D. This mutation was generated by PCR.

The luciferase reporter plasmids (4 × ABC)p95Luc, (4 × DE)p95Luc, (4 × ABC_C)_v95Luc, and (4 × DEF)_v95Luc were all constructed using the pLuc4 vector (6). Intermediate construction steps and multimerization of the enhancer elements were performed in the pS9Col2a1Bsl plasmid as described previously for other constructs (6). These plasmids contain four tandem copies of the A/B/C, D/E, A/B/Cm, or DEFm Col11a2 elements (as described in Fig. 1) cloned directly upstream of a minimal 89-bp Col2a1 promoter (−89 to +6).

The plasmids (4 × ABC)p95lacZ and (4 × DE)p95lacZ were constructed by cloning four copies of A/B/C or D/E directly upstream of a minimal human β-globin promoter (−44 to +28) in the reporter plasmid placZ as described previously for other constructs (20). DNA segments obtained by PCR or with synthetic oligonucleotides were verified by DNA sequencing.

SOX9 Antibody Purification—SOX9 antibodies were created by immunizing rabbits with a peptide (HSPQHWEQPVYTQLT), as described previously (21) (Genosys; The Woodlands, TX). The antibodies were affinity purified over a 3 M Emphaze Biosupport Medium AB1 column (Pierce) coupled to the SOX9 peptide and were eluted at low pH (22). They were then diluted in phosphate-buffered saline with 0.02% sodium azide and concentrated using Microcon-10 microconcentrators (Millipore, Bedford, MA). In electrophoretic mobility shift assay (EMSA) experiments, the purified SOX9 antibodies completely and specifically supershifted a SOX9-DNA complex formed between crude RCS nuclear extracts and a probe called R2, which is an 18-bp subfragment of the Col2a1 48-bp enhancer containing the SOX9-binding site. This probe was previously shown to form a major protein-DNA complex with Sox9 in RCS nuclear extracts (7).

In Vivo Synthesis of SOX9—SOX9 was synthesized by in vitro transcription/translation using the Single Tube Protein System 2, T7 kit from Novagen (Madison, WI) and a previously described expression plasmid containing the coding sequence for human SOX9 (7).

Electrophoretic Mobility Shift Assays (EMSA)—All probes (described above) were radiolabeled by end-filling with the Klenow fragment. Protein-DNA binding reactions were performed as described previously (6). In vitro synthesized SOX9 protein was assayed in the presence of 0.1 μg of poly(dG-dC)-poly(dG-dC) as nonspecific competitor. Nuclear extracts were prepared as described (23), and most assays were performed using 9 μg of protein and 1.0 μg of poly(dG-dC)-poly(dG-dC).

When SOX9 antibodies were included, they were coincubated with the protein and probe. Electrophoretic gel separations were usually run for 2–3 h at 160 V. The very long separations were run for 6–7 h at 160 V.

Transient Transfections—Transfections were performed using Lipo- fectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. A total of 2 μg of DNA was used to transfect each 10-cm2 dish. Cellular extracts were prepared and luciferase and β-galactosidase activities were assayed as described (18).

Generation and Analysis of Transgenic Mice—Prior to microinjections to make transgenic mice, pCXIwt and pCXImut were digested with EcoRI and PstI, and (4 × ABC)p95lacZ and (4 × DE)p95lacZ were digested with Asp718 and HindIII to remove extraneous plasmid sequences. Microinjections were performed as previously described (20). Founder embryos were sacrificed at 14.5 days postcoitum (dpc). Embryos were fixed and stained with X-gal, photographed as whole mounts, and then embedded and sectioned for histological analysis as described previously (20).

RESULTS

Mutation of the HMG Consensus Site Decreases Col11a2 Promoter Activity in Transient Transfections of Chondrocytes—The plasmid pCXIwt contains the 5′ region of Col11a2 from −742 bp to +380 bp, controlling a lacZ reporter gene. The pCXImut plasmid is identical in every way except that it contains a 2-bp mutation of the consensus HMG-binding site (site D, Fig. 1). Transient transfections of these two plasmids revealed that pCXIwt was very active in RCS cells, but mutation of the HMG consensus site in pCXImut reduced reporter gene activity to approximately one-third of pCXIwt levels (Fig. 2). Neither plasmid was active in BALB/3T3 fibroblasts, indicating that factors necessary for activation of this promoter may not be present in these cells. Cotransfection with a SOX9 expression vector did not measurably increase the activity of pCXIwt in fibroblasts (data not shown), suggesting that other factors besides SOX9 may be required to activate the −742 bp to +380 bp Col11a2 promoter in chondrocytes.

Mutation of the HMG Consensus Site Eliminated Cartilage-specific Col11a2 Promoter Activity in Transgenic Mice—DNA from the pCXIwt and pCXImut plasmids was used to generate transgenic mice. Founders were sacrificed at 14.5 dpc and stained with X-gal to identify embryos expressing lacZ. Of nine transgenic embryos harboring pCXIwt, seven displayed blue staining. In every positively stained mouse, the staining was primarily concentrated in the cartilage, including ribs, limbs,
Chondrocyte-specific Col11a2 Enhancer Elements

![Diagram](image)

**Fig. 1. Schematic representation of the 5′ region of Col11a2 and detail of DNA probes.** All nucleotide numbering is according to the sequence published by Tsumaki et al. (17), with +1 indicating the transcriptional start site and −742 indicating the putative 3′ end of the retinoic X receptor β gene which lies immediately upstream of Col11a2. Panel A, the relative positions of the five HMG-like sites, labeled A–E, are shown, and the alignment of the probes is indicated by filled bars. Panel B, the name of each probe is followed by its first and last nucleotides in parentheses. Subscript m in the name of a probe indicates that that particular HMG site is mutated in the probe. Lowercase letters in the probe sequences indicate flanking sequences that were added to facilitate radiolabeling and/or cloning. The HMG-like sites are shown in bold parentheses. The Consensus HMG Domain Protein Binding Site in the 5′ region of Col11a2 is critical for the chondrocyte-specific activity of the promoter in vivo.

The Consensus HMG Domain Protein Binding Site in the Col11a2 Promoter Binds Sox9—Given that Sox9 has previously been shown to activate transcription by binding a site in the 48-bp Col2a1 chondrocyte-specific enhancer which is homologous to the HMG binding consensus (7), EMSA experiments were performed to determine whether Sox9 could also bind to the Col11a2 HMG consensus site. The probe Dwt (Fig. 1) containing the consensus site and 10 bp of flanking DNA on either side was used for these assays. EMSAs showed that in vitro transcribed and translated Sox9 bound to the Dwt probe, forming two distinct DNA-protein complexes (Fig. 4, panel A). In addition to the D site, the Dwt probe also includes the E site is shown, and intensity observed here were comparable to that obtained by Tsumaki et al. (17) with this same Col11a2 construct.

Microinjection of the pCXImut construct produced seven 14.5 dpc transgenic embryos. Five of these showed weak promiscuous blue staining primarily in neural tissue or bone precursor, much like the weak nonspecific staining that was observed in addition to cartilage staining in pCXI wt mice. None of the pCXImut mice, however, had any staining in cartilage (Fig. 3, bottom row). This result indicates that the consensus HMG domain protein-binding site in the 5′ region of Col11a2 is critical for the chondrocyte-specific activity of the promoter in vivo.

**Fig. 2. Transient transfections demonstrate that the consensus HMG-binding site in the 5′ region of Col11a2 is important for promoter activity.** The plasmid pCXIwt contained the 742-bp Col11a2 promoter controlling a lacZ reporter gene, and pCXImut was identical except for a 2-bp mutation within the consensus HMG-binding site. These plasmids were transiently transfected into chondrocytic RCS cells and into BALB/3 T3 fibroblasts. Experiments included 1.5 μg of reporter plasmid and 0.5 μg of an internal control for transfection efficiency, the pGL2-Control vector which expresses luciferase (Promega; Madison, WI). Results are presented as β-galactosidase units per luciferase unit ± S.D. The graph displays data from one representative experiment performed in triplicate.
The protein complexes contain very similar or the same proteins as (28). Several lines of evidence suggest that the Col11a2 complex have been tentatively designated CSEP by Zhou et al. (28) have also concluded that the Col11a2 A/B/C and D/E probes were eliminated by the use of poly(dI-dC) as a nonspecific competitor in the EMSA experiments (Fig. 6). Third, neither the Col2a1 nor the Col11a2 probes formed these complexes with nuclear extracts from BALB/3T3 fibroblasts (Fig. 6). And fourth, the formation of the major complex between RCS cell nuclear extracts and each of the three probes was competed by a 10-fold excess of any of the other probes or of self, while other minor complexes formed by the three probes were not competed except by self (Fig. 7). In summary, these experiments support the hypothesis that the proteins present in the Col11a2 DNA-protein complexes and in CSEP are very similar.

The slowest complexes formed by in vitro made Sox9 with each of the three probes migrated as compact bands at the trailing boundaries of the CSEP-DNA complex and the CSEP-like complexes, even after very long electrophoretic separation, suggesting that Sox9 could be present in the complexes. The broad bands produced by long separation of the complexes, however, indicate that Sox9 is probably not the only protein present. Zhou et al. (28) have also concluded that the Col2a1 CSEP-DNA complex is heterogeneous. Inclusion of Sox9 antibodies in EMSA experiments with the Col11a2 probes and RCS cell nuclear extracts resulted in the appearance of a minor supershifted species, but the majority of each complex remained unmoved (Fig. 8). The experiment was performed under the same conditions in which the antibodies completely supershifted a Sox9 complex with an 18-bp Col2a1 probe (called R2) in RCS nuclear extracts (Ref. 7 and data not shown). These results support the conclusion that Sox9 may constitute the slower migrating portion of the CSEP-like complexes but is not the only protein in the complex.

Four of the Five Putative HMG Sites Participate in Binding of the CSEP-like Proteins—DNA probes were made that contained mutations designed to eliminate the potential HMG domain protein-binding sites in probes A/B/C and D/E (Fig. 1). In EMSAs with RCS nuclear extracts, mutation of sites C, D, or E completely eliminated formation of the CSEP-like complex. Mutation of site B greatly reduced complex formation, whereas mutation of site A had no effect (Fig. 9). The observation that the single mismatch HMG site A was not involved in complex formation but that the double mismatch site B was necessary reveal any differences in the electrophoretic mobilities of the three complexes (Fig. 5, lanes 2, 4, and 6). Second, like the Col2a1 CSEP-DNA complex, the protein-DNA complexes formed with the Col11a2 A/B/C and D/E probes were eliminated by utilizing the multiple HMG-like binding sites.

DNA probes were constructed, one included sites A, B, and C and the other included sites D and E (Fig. 1). They were used in EMSA analysis with in vitro synthesized Sox9, and both formed a major complex and a minor, faster migrating complex (Fig. 5, lanes 3 and 5, arrows). Two complexes with the same mobility were observed when the Col2a1 48-bp enhancer probe was used, but the faster migrating complex was much stronger (Fig. 5, lane 1). Both complexes with all three probes were completely supershifted by antibodies to Sox9 (data not shown), and we postulate as with the Dwt probe that Sox9 forms both monomeric and dimeric complexes with the probes by utilizing the multiple HMG-like binding sites.

In EMSA experiments with RCS cell nuclear extracts, the major DNA-protein complex formed by the Col11a2 A/B/C and D/E probes (Fig. 5, lanes 4 and 6, bracket) was similar in mobility to the chondrocyte-specific complex formed with the 48-bp Col2a1 enhancer (Fig. 5, lane 2). The proteins in this complex have been tentatively designated CSEP by Zhou et al. (28). Several lines of evidence suggest that the Col11a2 DNA-protein complexes contain very similar or the same proteins as the Col2a1 CSEP-DNA complex. First, very long separation (2–3 times longer than usual) EMSA experiments failed to
suggests that the position of the HMG-like sites in relation to each other plays an important role in protein binding. Sites B and C are separated by 4 bp and sites D and E are separated by 3 bp. Site A, conversely, is 18 bp upstream of B.

The Sites That Bind CSEP-like Proteins Are Necessary for Enhancer Activity in Transient Transfections—

The plasmids (4×ABC)p95Luc and (4×DE)p95Luc contain four tandem copies of the A/B/C or D/E sequences inserted upstream of a minimal 89-bp Col2a1 promoter, controlling a luciferase reporter gene. This minimal promoter was chosen because it has previously been shown to be essentially inactive by itself but strongly activated in chondrocytes by four copies of the 48-bp Col2a1 enhancer (6). In transient transfection experiments, four copies of the A/B/C or of the D/E elements were active in RCS cells. Activity of the A/B/C element was approximately 1,000 times higher than the almost negligible p95Luc background levels, and the D/E enhancer was five times more active than the A/B/C enhancer (Fig. 10, panel A). Mutation of the sites that participated in binding of the CSEP-like complexes in vitro made SOX9 did not broaden after long separation.

EMSAs experiments in plasmids (4×ABC)p95Luc and (4×DE)p95Luc abolished virtually all enhancer activity of both elements, indicating that these sites are critical for this activity (Fig. 10, panel A). The non-mutated enhancer constructs were no more active than the enhancerless p95Luc plasmid in BALB/3T3 fibroblasts, but cotransfection of a SOX9 expression vector with the plasmids resulted in approximately 5-fold activation (Fig. 10, panel B).

Four Copies of Either Enhancer Sequence Target Reporter Gene Expression to Many Cartilages of Transgenic Mice—

Four copies of the A/B/C sequence or of the D/E sequence were cloned upstream of a heterologous minimal human β-globin promoter, with a lacZ reporter gene. When microinjected into fertilized mouse eggs, the D/E construct produced 9 mice that were.
positive for the transgene as determined by Southern hybridization. Four of these displayed weak reporter gene expression. The expression was present in the cartilage of the limbs, digits, ribs, nasal septum, mandible, and trachea but not in vertebrae or in non-cartilaginous tissues (Fig. 11, panels b and e–h). Four copies of the A/B/C sequence with the minimal human β-globin promoter produced 10 transgenic mice, of which one displayed reporter gene expression. The transgene was expressed weakly in the cartilage of the limbs, digits, and ribs but not in other cartilage or in non-cartilaginous tissues (Fig. 11, panels a, c and d). These results suggest that the A/B/C and D/E sequences of the Col11a2 gene are capable of directing weak but specific transgene expression to many cartilaginous structures of developing mice.

**DISCUSSION**

Type XI collagen is a critical component of cartilage collagen fibrils, and studies have shown that mutations in the genes for two of its subunits, Col11a1 and Col11a2, cause skeletal malformation by producing abnormal cartilage (1, 2). The present study focused on the 5′-regulatory region of the Col11a2 gene, which includes elements that participate in directing transgene expression to cartilage in transgenic mice (17). Within this region lies a consensus HMG domain protein-binding site. This site is of particular interest because the HMG domain-containing protein SOX9 is believed to play an important role in chondrogenesis, based on evidence that the severe skeletal malformation syndrome campomelic dysplasia is caused by mutations in the SOX9 gene (8–10). In the present study, a mutation of 2 bp within this HMG consensus site in the Col11a2 promoter greatly decreased binding of SOX9 in EMSA, markedly reduced promoter activity in transient transfections in chondrocytes, and eliminated all chondrocyte-specific Col11a2 promoter activity in transgenic mouse embryos, suggesting that this site is critically important.

Type II collagen is also a critical component of cartilage collagen fibrils (3–5, 24, 25), and we have previously identified a 48-bp chondrocyte-specific enhancer in its Col2a1 gene which binds SOX9 and is activated by it (6, 7). The accompanying paper (28) reports the mutational analysis of two HMG-like binding sites in addition to the SOX9-binding site in the 48-bp Col2a1 enhancer, demonstrating that these sites are also necessary for chondrocyte-specific enhancer activity in transient transfections and in transgenic mice. These sites participate in the formation of a chondrocyte-specific DNA-protein complex, the protein components of which were designated CSEP and are believed to include SOX9 and other proteins with similarities to the HMG protein family (28). The Col11a2 gene also contains several sites with homology to the HMG domain-binding consensus sequence within its promoter region, and so two DNA probes named A/B/C and D/E were constructed which spanned these sites, to determine whether they also form chondrocyte-specific DNA-protein complexes.

When the two DNA probes were used in EMSA experiments with *in vitro* synthesized SOX9, they each formed SOX9-DNA complexes that were almost completely supershifted by antibodies to SOX9. When these probes were used in EMSA experiments with crude nuclear extracts from the chondrocytic cell line RCS, however, they formed protein-DNA complexes that could be only partially supershifted by antibodies to SOX9. The complexes had the same mobility as the 48-bp Col2a1 enhancer-CSEP complex described in the accompanying paper (28) and were not formed with fibroblast nuclear extracts. After long-run EMSA, the Col11a2 complexes spread to form broad heterogeneous bands that remained identical to the Col2a1 CSEP-DNA complex. Each complex was sensitive to competition by the other two probes and by poly(dI-dC). Together, these experiments support the hypotheses that Sox9 is present in the complexes formed with all three probes and that additional protein(s) in the three complexes are very similar if not identical.

Zhou et al. (28) present experiments strengthening the argument that the CSEP complex contains proteins in addition to SOX9. RCS nuclear extracts were fractionated by SDS-polyacrylamide gel electrophoresis and then eluted from gel slices. The eluted and renatured proteins were subjected to EMSA with the 48-bp Col2a1 enhancer probe, and a complex formed with eluates containing proteins with an apparent molecular mass of 75–95 kDa. The apparent molecular mass of SOX9 had previously been shown to be about 68 kDa (4). The complex that formed with the 75–95-kDa proteins had a similar mobility to the CSEP-DNA complex and was not supershifted by SOX9 antibodies. In addition, Zhou et al. (28) report that no CSEP complex formed between the 48-bp Col2a1 enhancer and 10T1/2 fibroblast nuclear extracts, although these cells are known to contain SOX9.

Zhou et al. (28) also demonstrated that formation of the 48-bp Col2a1-CSEP complex was dependent on the presence of HMG-like sites and that these sites were necessary for en-
hancer activity in transient transfection experiments and in transgenic mice. We have herein demonstrated that the B and C sites in the A/B/C probe and the D and E sites in the D/E probe are similarly essential for CSEP-like protein DNA complex formation. The A site, however, is not. This result was somewhat surprising because the A site is a 1-bp mismatch HMG site, whereas the B site is a 2-bp mismatch. The A site is separated from the others by 18 bp, whereas sites B and C and sites D and E occur in pairs separated by 4 or 3 bp, respectively. The fact that the A site is more homologous with the HMG consensus than the B site and yet plays no role in binding of the CSEP-like proteins suggests that the arrangement of the binding sites in clusters of at least two sites may be critical to their function. Interestingly, the Col2a1 48-bp chondrocyte-specific enhancer contains at least three HMG-like sites in close proximity to one another. Understanding the significance of this spatial arrangement of HMG-binding sites will require more study, but it seems possible that clustered sites may be required to bind the multiple proteins in CSEP. This might explain the tendency of in vitro synthesized SOX9 to form dimeric as well as monomeric complexes with the Col11a2 and Col2a1 probes. Alternatively, a protein in CSEP may have more than one HMG domain and consequently require adjacent HMG-binding sites.

The evidence discussed thus far is consistent with the hypothesis that the A/B/C and D/E portions of the Col11a2 gene bind proteins (including Sox9) that are the same as or at least similar to those that bind the 48-bp Col2a1 chondrocyte-specific enhancer. Transient transfection experiments revealed that the Col11a2 sequences also have chondrocyte-specific enhancer activity like the 48-bp Col2a1 element and that the sites involved in CSEP-like complex formation are essential for enhancer activity. Interestingly, the B, C, and E sites that participate in CSEP-like complex formation are completely conserved in the human Col11a2 gene, and the perfect consensus D site that also participates in complex formation differs at only one base. The A site, however, plays no role in binding of the CSEP-like proteins and differs at three of its seven bases (26). Although the A/B/C and D/E enhancer elements were inactive in fibroblasts, cotransfection with a SOX9 expression plasmid activated both enhancers as it has been shown previously to activate the 48-bp Col2a1 enhancer, suggesting that SOX9 may act upon these chondrocyte-specific enhancers in vivo (7). The activation, however, was relatively weak compared with the activity of the enhancers in RCS cells. SOX9 activation of the enhancers in fibroblasts produced reporter gene expression levels that were approximately 5–10 times higher than base-line p95Luc levels, whereas the same enhanc-

FIG. 10. The A/B/C and D/E elements have chondrocyte-specific enhancer activity in transient transfections, and the sites that bind CSEP-like proteins are necessary for that activity. Panel A, four tandem copies of the A/B/C or D/E probes, either wild-type or carrying mutations at all the sites shown in Fig. 9 to be important for CSEP-like protein binding, were cloned as enhancer elements in the luciferase expression vector p95Luc. The plasmids were transiently transfected into RCS cells. Each transfection included 1.5 μg of reporter plasmid and 0.5 μg of an internal control for transfection efficiency. Panel B, the wild-type enhancer plasmids that were active in RCS cells (panel A) were transiently transfected into BALB/3T3 fibroblasts either with or without cotransfection of a SOX9 expression vector. Note that the enhancers were no more active than the p95Luc base line in this cell line but were activated approximately 5-fold by cotransfection with SOX9. Each transfection included 1.0 μg of reporter plasmid, 0.5 μg of SOX9 expression plasmid, and 0.5 μg of internal control. In both sets of experiments, the internal control was the pSV-β-galactosidase vector (Promega; Madison, WI). Results are presented as 10^4 ± luciferase units per β-galactosidase unit ± S.D. Each panel shows data from two independent experiments, performed in triplicate.
ers in RCS cells were roughly 1,000 and 5,000 times more active than p95Luc. This suggests that RCS cells contain other factors in addition to Sox9 that are necessary to achieve full activation of the enhancers.

Eight tandem copies of the 48-bp Col2a1 enhancer, when paired with a heterologous promoter, are capable of directing transgene expression to all cartilaginous structures of a developing mouse (28). In the present study we have shown that four tandem copies of the A/B/C or D/E enhancers, when paired with the same heterologous promoter and used to generate transgenic mice, are similarly sufficient to direct chondrocyte-specific expression in a whole animal. The D/E element, which was most active in transient transfection experiments, directed low level transgene expression to the cartilage of ribs, limbs, digits, and nasal septum (×10; e), forelimb digits (×10; f), and hindlimb digits (×10; d).

The A/B/C and D/E enhancers can direct chondrocyte-specific expression in transient transfection and in transgenic mouse embryos. Like the Col2a1 enhancer, they contain clustered HMG-type protein-binding sites that are essential for their enhancer activity and that bind proteins in vitro which are similar and perhaps identical to the CSEF proteins that bind to the Col2a1 enhancer in vitro. It is possible that the same proteins play a role in the chondrocyte-specific activation of these enhancers in vivo.

The Col2a1 and Col11a2 enhancers are both necessary for the normal development of cartilage. The presence of parallel enhancer elements in these two critical genes suggests that they may both be regulated by the same mechanism during chondrogenesis, perhaps as part of a larger genetic program designed to efficiently coordinate the expression of multiple genes involved in chondrocyte differentiation.

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