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An 18-Base-Pair Sequence in the Mouse Proα1(II) Collagen Gene Is Sufficient for Expression in Cartilage and Binds Nuclear Proteins That Are Selectively Expressed in Chondrocytes

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The molecular mechanisms by which mesenchymal cells differentiate into chondrocytes are still poorly understood. We have used the gene for a chondrocyte marker, the proα1(II) collagen gene (*Col2a1*), as a model to delineate a minimal sequence needed for chondrocyte expression and identify chondrocyte-specific proteins binding to this sequence. We previously localized a cartilage-specific enhancer to 156 bp of the mouse *Col2a1* intron 1. We show here that four copies of a 48-bp subsegment strongly increased promoter activity in transiently transfected rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not in 10T1/2 fibroblasts. They also directed cartilage specificity in transgenic mouse embryos. These 48 bp include two 11-bp inverted repeats with only one mismatch. Tandem copies of an 18-bp element containing the 3′ repeat strongly enhanced promoter activity in RCS cells and chondrocytes but not in fibroblasts. Transgenic mice harboring 12 copies of this 18-mer expressed luciferase in ribs and vertebrae and in isolated chondrocytes but not in noncartilaginous tissues except skin and brain. In gel retardation assays, an RCS cell-specific protein and another closely related protein expressed only in RCS cells and primary chondrocytes bound to a 10-bp sequence within the 18-mer. Mutations in these 10 bp abolished activity of the multimerized 18-bp enhancer, and deletion of these 10 bp abolished enhancer activity of 465- and 231-bp intron 1 segments. This sequence contains a low-affinity binding site for POU domain proteins, and competition experiments with a high-affinity POU domain binding site strongly suggested that the chondrocyte proteins belong to this family. Together, our results indicate that an 18-bp sequence in *Col2a1* intron 1 controls chondrocyte expression and suggest that RCS cells and chondrocytes contain specific POU domain proteins involved in enhancer activity.

Acquisition of the chondrocyte phenotype by mesenchymal cells is one of the major pathways of differentiation of these cells. Chondrocytes form several types of cartilages including the growth plate cartilages essential to skeletal formation and cartilages that have supporting roles and persist throughout adult life such as the articular cartilages and the cartilages of the nose, ear, and trachea. Chondrocyte differentiation presumably involves first the commitment of undifferentiated mesenchymal cells to the chondrocyte lineage (1). Cell condensation and further maturation lead to a fully differentiated phenotype characterized by the synthesis of cartilage extracellular matrix proteins, including collagen types II, IX, and XI, the large proteoglycan aggrecan, the link protein, and the cartilage oligomeric protein (24). Recent molecular and biochemical studies with cell culture, gene inactivation experiments with mice, and the identification of genes responsible for mouse and human skeletal abnormalities have documented the importance of growth and differentiation factors, extracellular matrix proteins, signaling mediators, and transcription factors in skeletal development (5, 23). However, no specific transcription factors that control the differentiation of chondrocytes from mesenchymal cells and activate chondrocyte-specific genes have yet been identified.

Type II collagen is the most abundant extracellular protein made by chondrocytes. Its essential structural role in cartilage is best illustrated by the severe skeletal anomalies shown by humans and mice carrying mutant proα1(II) collagen chains (7, 25). The type II collagen gene starts to be expressed following mesenchymal cell condensation that precedes cartilage formation, and thus it represents an early marker of chondrocyte differentiation. The type II collagen gene is also expressed transiently in some extrachondrogenic sites during embryonic development, including the notochord, heart, epidermis, and discrete areas of the brain (2). However, expression at these sites is low, and the role of type II collagen in these extrachondrocytic sites is not understood. The type II collagen gene should therefore be an excellent model for studies of chondrocyte-specific transcriptional mechanisms.

Previous DNA transfection studies showed that 620 bp of the first intron of the rat Col2a1 gene enhanced promoter activity specifically in primary chick chondrocytes (11). Later, studies of this enhancer pinpointed a 260-bp sequence that enhanced promoter activity sixfold in chondrocytes (26). In another study, two silencer elements were located in the rat *Col2a1* promoter and were proposed to inactivate the gene in nonchondrocytic cells (21). Experiments with transgenic mice indicated that the first intron of the rat Col2a1 gene was necessary to direct cartilage-specific activity of a 3-kb *Col2a1* promoter (27). In another study, the DNA extending from 3 kb upstream of the start of transcription to exon 4 of the mouse Col2a1 gene conferred a pattern of *lacZ* expression in transgenic mouse embryos that coincided with the chondrocytic

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expression of the endogenous gene during embryonic development, and deletion of intron 1 inhibited expression of the transgene (15).

Our laboratory has recently started to reexamine the *cis*-acting elements that direct expression of the mouse Col2a1 gene in chondrocytes. In one approach we generated and studied transgenic mice (28), and in another, parallel approach we performed transient expression experiments in rat chondrosarcoma (RCS) cells, mouse primary chondrocytes, and, as controls for nonchondrogenic cells, 10T1/2 fibroblasts and C_2C_{12} myoblasts (17). RCS cells are a stable and fully differentiated chondrocyte cell line that synthesizes type II, IX, and XI collagens as well as cartilage-specific proteoglycans (17). Unlike other so-called chondrocyte cell lines, they contain no type I collagen RNA. They also contain no type X collagen RNA, suggesting that they were frozen in a stage of chondrocyte differentiation that precedes hypertrophy. Analysis of progressively shorter *Col2a1* intron 1 segments revealed that two tandem copies of a 182-bp fragment were sufficient for cartilage expression in transgenic mice and that two tandem copies of a 156-bp fragment, included in the 182 bp, were able to strongly increase promoter activity in RCS cells but not in 10T1/2 fibroblasts. Further deletions suggested that the 3′ and 5′ parts of the 156- and 182-bp segments might be necessary for chondrocyte expression. We also determined that the Col2a1 promoter was dispensable for chondrocyte expression (17, 28). Indeed, strong promoter activation was still obtained in chondrocytes in both transgenic mice and transient transfections when a 182-bp enhancer or a 231-bp intron fragment containing the 182 bp was cloned upstream of a minimal heterologous promoter, either a β-globin promoter or the adenovirus major late promoter.

In the present study, we aimed at delineating more precisely the *cis*-acting elements in *Col2a1* intron 1 that are needed for chondrocyte expression and also asked whether chondrocyte-specific proteins were binding to these elements. We show that multiple copies of an 18-bp subsegment of the 156-bp enhancer can strongly enhance promoter activity selectively in transiently transfected RCS cells and chondrocytes and are also sufficient to direct promoter activity in chondrocytes of transgenic mice. Evidence that nuclear proteins present selectively in primary chondrocytes and RCS cells bind within this 18-mer to a 10-bp sequence which is essential for enhancer activity and that these proteins likely belong to the POU domain protein family is presented.

MATERIALS AND METHODS

Cell cultures. RCS cells were given by J. H. Kimura (Henry Ford Hospital, Detroit, Mich.), and ROS 17/2.8 cells were given W. T. Butler (The University of Texas Health Science Center, Houston). Rib chondrocytes were isolated from newborn mice as previously described (14). Other cell lines were from the American Type Culture Collection (Rockville, Md.). Cells were cultured under standard conditions (17).

Transient transfections. DNA transfections were carried out as described previously (17). Luciferase reporter plasmids were cotransfected with the pSV2βgal plasmid used as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were assayed as described elsewhere (17). Differences between several experiments in the values obtained for a given construction transfected in a given cell type can be explained by assay variations from one experiment to another. Luciferase activities were expressed as 2×10^4 luciferase units per β-galactosidase unit. Luciferase values in extracts of RCS cells transfected with the 89-bp *Col2a1* promoter alone were never more than twice the blank; values with active enhancer elements were between 50- and 20,000-fold higher depending on the constructions used.

Col2a1-luciferase constructions. All final Col2a1 constructions were cloned in the pLuc4 vector (18). For intermediate construction steps, *Col2a1* intron 1 segments were cloned in the p89Col2a1Bs plasmid. This vector was obtained by cloning the 89-bp *Col2a1* promoter (−89 to +6) containing blunt-ended *Hin*dIII sites at both ends (17) between the *Eco*RV site and the blunt-ended *Xho*I site of pBluescript II Ks (+/−) (Stratagene, La Jolla, Calif.). Ligation of blunt-ended *Hin*dIII and *Xho*I sites reconstituted a *Hin*dIII site 3′ of the promoter. Single or tandem copies of intron 1 segments were cloned together with the 89-bp *Col2a1* promoter upstream of the luciferase gene in pLuc4.

Single copies or duplicate tandem copies of *Col2a1* intron 1 segments of 156, 231, and 465 bp were obtained as previously described (17). Other intron 1 segments were synthesized as double-stranded oligonucleotides containing a *Bam*HI site at the 5′ end and a *Bgl*II site followed by an *Eco*RI site at the 3′ end. These oligonucleotides were cloned between the *Bam*HI and *Eco*RI sites of p89Col2a1Bs. Dimers were obtained by cloning a second oligonucleotide molecule in the *Bgl*II and *Eco*RI sites. Tetramers were obtained by releasing the two oligonucleotide copies by *Bam*HI and *Eco*RI digestion and inserting them in a vector containing two copies and cut at the *Bgl*II and *Eco*RI sites. Further multimerization was done as for tetramers.

Plasmid p41Col1a2, originally called pH39 (8), contained a minimal *Col2a1* promoter cloned between the *Asp*718 and *Hin*dIII sites of pA_3LUC. Twelve copies of the R2 segment of *Col2a1* were introduced in this vector between the *Spe*I and *Bam*HI sites.

A 10-bp deletion within *Col2a1* intron 1 fragments was obtained by PCR. All constructions made with oligonucleotides and products of PCR were verified by DNA sequencing.

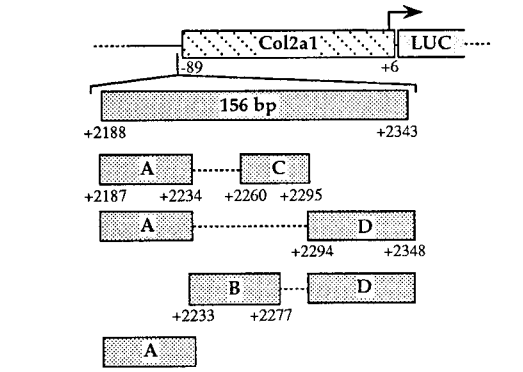
Transgenic mice. Two DNA constructions were made by cloning a four-copy A element or a 12-copy R2 element as a blunt-ended *Bam*HI-*Bgl*II fragment in the blunt-ended *Spe*I site of p309Col2a1 (28). This vector contained a 309-bp *Col2a1* promoter and the SA-βgeo-bpA cassette (6). For a third construction, the 12-copy R2 element was cloned upstream of an 89-bp *Col2a1* promoter in the pLuc4 vector. DNAs were released by restriction enzyme digestion 5′ of *Col2a1* sequences and 3′ of the polyadenylation signal located downstream of the reporter genes. Transgenic mice harboring these DNAs were generated as described elsewhere (28). Transgenic founder mice were sacrificed at day 14.5 of embryonic development or within 3 days after birth. Southern analysis, staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), measurements of luciferase activity in tissue extracts, and histology studies were performed as previously described (18, 28).

Nuclear extracts. Nuclear extracts from mouse chondrocytes were prepared as described elsewhere (4), either directly after isolation of the cells from cartilage or after up to 3 days in primary culture. One day before harvest of primary cells, ascorbic acid was added to culture media (17). Nuclear extracts from all other cell types were prepared as previously described (3) with 10 μg of leupeptin and pepstatin per ml in all buffers.

Gel retardation assays. The wild-type and mutant R2 probes were made by annealing complementary oligonucleotides as described in the legend to Fig. 5A. The OCT probe was made with oligonucleotides (5′-ggCCTGGGTAATTTGCATTTCTAAAA-3′ and 5′-ggTTTTAGAAATGCAAATTACCCAGG-3′) corresponding to a fragment of the immunoglobulin heavy-chain gene enhancer which contains an octamer binding site for POU domain proteins (22). G residues were added at the 5′ ends for labeling.

Protein-DNA binding reactions were carried out with 10 fmol of a ^{32}P-end-labeled probe in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 50 mM KCl, 10% (vol/vol) glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% (vol/vol) Nonidet P-40, 50 μg of bovine serum albumin (BSA), and 4 pmol of one of the two single-stranded R2 oligonucleotides. Assays with crude nuclear extracts were performed with 10 to 15 μg of protein and 0.5 μg of poly(dI-dC) · poly(dI-dC) plus, in some cases, 0.5 μg of poly(dG-dC) · poly(dG-dC). Purified proteins were assayed in the absence of a nonspecific DNA competitor. In supershift experiments, antibodies were added just before nuclear proteins. An antiserum containing Oct-1 antibodies was provided by M. Perry (The University of Texas Southwestern Medical Center, Dallas) (10). Polyclonal antibodies against Oct-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Reaction mixtures (25 to 30 μl) were incubated for 30 min at room temperature and fractionated on a 4% (wt/vol) polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) for 3.5 to 4 h at 150 V.

Purification and characterization of RCS cell-specific DNA-binding proteins. Nuclear extracts from RCS cells (about 400 mg of protein) were diluted at 2 mg/ml in buffer A (20 mM HEPES [pH 7.9], 10% [vol/vol] glycerol, 1 mM EDTA, 0.05% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of leupeptin and pepstatin per ml) supplemented with 70 mM NaCl. Extracts were loaded on a first 7-ml DNA affinity column which was prepared by the method described in reference 12 by covalent coupling of the wild-type double-stranded R2 oligonucleotide (see Fig. 5A) to CNBr-activated Sepharose 4B (Sigma, St. Louis, Mo.). After successive washes of the column with buffer A containing 70 and then 150 mM NaCl, chondrocyte-specific proteins were eluted in buffer A supplemented with 300 mM NaCl. The eluted fraction was diluted in 3 volumes of buffer A and loaded on a second DNA affinity column of about 6 ml. This column contained a highly mutated enhancer fragment made with complementary oligonucleotides (5′-gatccAAAGCCCGTTCTACAGCAtctg-3′ and 5′-aattcagaTGCTGTAGAACGGGCTTTg-3′). Chondrocyte-specific proteins were eluted from this column at 150 mM NaCl. Fractions were diluted to 125 mM NaCl and loaded on a 1-ml Mono Q column (Pharmacia Biotech, Piscataway, N.J.). Chondrocyte-specific proteins were recovered in the flowthrough, which was then diluted to 50 mM NaCl and applied to a 1-ml Mono S column (Pharmacia). Proteins were eluted by using a 10-ml



B

Intron-1 Segments	RCS	10T1/2
none	0.2 ± 0.3	0.7 ± 0.2
2 x 156	29.6 ± 6.4	2.4 ± 0.8
1 x AC	0.2	1.5
2 x AC	70.1 ± 29.7	1.6 ± 0.3
1 x AD	2.9	5.3
2 x AD	467 ± 143	5.9 ± 1.4
2 x BD	0.3	3.85
2 x A	0.0	1.8
4 x A	1478 ± 112	2.3 ± 1.0

C

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mouse   CTGTGAATCGGGCTCTGTATGCGCTTGAGAAAAGCCCCATTCATGAGA
rat     ........................C.....................
human   ..................G......C.....................
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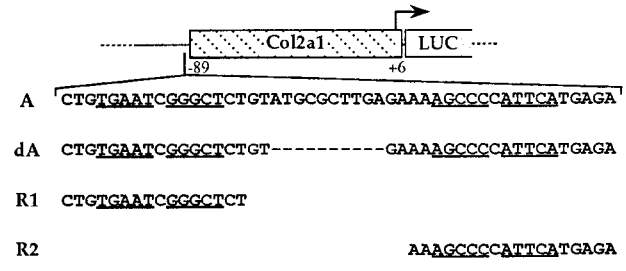
D



E

Intron-1 Segments	RCS (1)	RCS (2)	10T1/2
none	0.2	0.2	1.5
4 x A	1457	219	0.8
4 x dA		325	9.7
8 x R1	0.1		1.4
3 x R2		0.66	1.4
4 x R2		3.5	
6 x R2		74.9	2.3
8 x R2		469	
12 x R2		1157	4.5

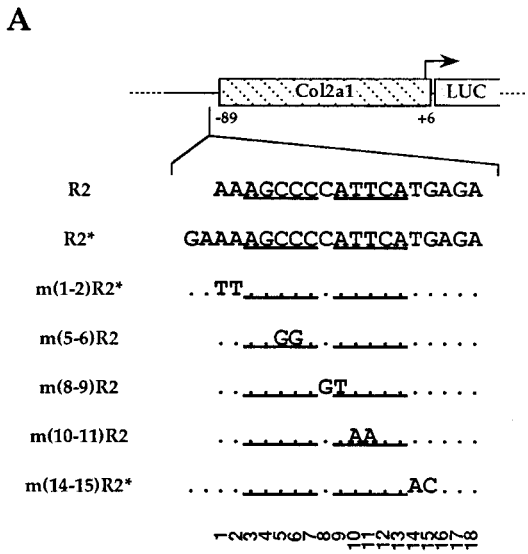
FIG. 1. Delineation of an 18-bp *Col2a1* enhancer. (A) DNA constructions. The 156-bp enhancer segment of *Col2a1* intron 1 and subsegments of this enhancer, designated A, B, C, and D, were cloned individually or in combinations (indicated by dotted lines) as one copy or two or four tandem copies upstream of an 89-bp *Col2a1* promoter driving the luciferase (LUC) gene. Numbers indicate the distance of the first and last nucleotides of each segment from the transcription start site in the Col2a1 gene. (B) Transient transfection experiments with RCS and 10T1/2 cells and the constructions shown in panel A. The intron 1 segments and the number of tandem copies of these segments in each construction tested are indicated in the first column. Luciferase activities are shown as the average values ± standard deviations for two to four independent cultures tested in one or two representative experiments. (C) Nucleotide sequence of the A element. The coding strand of the mouse *Col2a1* A element is aligned with the analogous region in the human and rat genes. Only bases in the human and rat genes that differ from the mouse sequence are shown; identical bases are indicated by dots. The two inverted repeats are indicated by arrows, and their nucleotides are underlined except at one G/C mismatch. (D) Constructions made with subsegments of the A element. The sequences of subsegments dA, R1, and R2 are aligned with that of the A element. The 10-bp deletion from A in dA is represented by dashes. Nucleotides of the two repeats are underlined, except for one G/C mismatch. All elements were cloned as multiple tandem copies in the same vector as in panel A. (E) Transient transfection experiments in RCS and 10T1/2 cells. DNA constructions were made as described for panel A by using 3 to 12 tandem copies of the elements shown in panel D. Luciferase activities are shown as averages for duplicate cultures in two representative experiments with RCS cells and one experiment with 10T1/2 cells.

NaCl concentration gradient from 50 to 300 mM, followed by 4 ml of 1 M NaCl. Chondrocyte-specific proteins were eluted at 150 to 250 mM salt.

Protein purification was monitored by gel retardation assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% polyacrylamide gels. Silver staining of proteins in gels was done by the method described in reference 16, with 100 μl of Mono S fractions concentrated by precipitation with trichloroacetic acid. Protein standards were obtained from Amersham (Arlington Heights, Ill.). For protein elution-renaturation experiments, 30-μl samples of Mono S fractions were denatured at 100°C in the SDS-PAGE sample buffer. After electrophoresis, gels were extensively washed for 1 h in buffer A and cut into slices. Each slice was crushed, and proteins were eluted in 4 volumes of buffer A supplemented with 50 mM KCl and 2.5 mg of BSA per ml for 3 h at room temperature. Gel shift assays were done by adding the labeled probe to 25 μl of eluates.

RESULTS

Delineation of an 18-bp *Col2a1* enhancer in RCS cells. Results obtained in our previous studies were consistent with the notion that elements in the 5′ and 3′ parts of a 156-bp *Col2a1* intron 1 segment could be needed together to generate enhancer activity in chondrocytes (17, 28). To better delineate active enhancer elements, we divided this 156-bp segment into four subfragments (Fig. 1A). The 5′ A element contained two inverted repeats of 11 bp each with one mismatch, separated by 18 bp. This element is highly conserved among the human, rat, and mouse genes (Fig. 1C) (13, 19). The 3′ C and D elements



B

Intron Segments	Expt 1	Expt 2
none	0.68	0.38
12 x R2	3594	2805
12 x R2*	6050	
12 x m(1-2)R2*	1.21	
12 x m(5-6)R2		3893
12 x m(8-9)R2	0.85	
12 x m(10-11)R2	0.85	0.11
12 x m(14-15)R2*	4.68	

FIG. 2. Transcriptional activity of mutant R2 and R2* elements. The sequence of the R2 element is aligned with that of a 20-bp R2* element containing the two additional nucleotides present on the 5′ side in the *Col2a1* sequence and with those of R2 and R2* mutants. The nucleotides of the R2 element were numbered 1 to 18 from the 5′ to the 3′ end. Only mutated nucleotides are shown; unchanged nucleotides are represented by dots. Positions of mutated nucleotides are indicated in parentheses in the designations of the mutants. Nucleotides corresponding to the 3′ repeat in element A are underlined, except at a G/C mismatch. These wild-type and mutant intron 1 segments were each cloned as 12 tandem copies in the vector in Fig. 1. Constructions were transfected transiently in RCS cells. Luciferase activities are presented as averages for two (experiment 1) or three (experiment 2) cultures in representative experiments. Note that the R2* element appears to be more active than the R2 element in experiment 1 but on average the two elements were similarly active. LUC, luciferase.

each corresponded to a sequence that showed DNase I protection with nuclear extracts of both RCS cells and 10T1/2 fibroblasts (17). The B element partially overlaps A and C. Various combinations of these elements were cloned upstream of an 89-bp *Col2a1* promoter driving the luciferase reporter gene, and the constructions were tested in transient transfections in RCS and 10T1/2 cells (Fig. 1A and B). As observed previously (17), the promoter by itself was barely active in the two cell lines, but it was highly activated in RCS cells and only minimally activated in 10T1/2 fibroblasts by two copies of the 156-bp enhancer. One copy of either AC or AD was essentially inactive in both cell types, but two copies of either combination strongly stimulated promoter activity in RCS cells but not in fibroblasts (Fig. 1B). Two copies of BD and two copies of A alone were essentially inactive in both cell types. Four tandem copies of A alone induced very strong promoter activation in RCS cells, reaching a level about 50-fold higher than that achieved by two copies of the 156-bp enhancer. Activation was minimal in fibroblasts. We concluded that the 48-bp A element contained the *cis*-acting sequences responsible for the RCS cell specificity of the *Col2a1* enhancer and that the C and D segments might contain binding sites for proteins that cooperated with factors binding to A in order to generate a high level of enhancer activity in RCS cells. Although D was more potent than C, these two segments appeared to play similar roles and were not necessary together.

We then tested whether the two inverted repeats in A and their 18-bp linker were necessary for enhancer activity (Fig. 1D and E). Four copies of an element harboring a 10-bp deletion within the linker (dA) activated the 89-bp *Col2a1* promoter in RCS cells as efficiently as A. Eight copies of an element containing the 5′ repeat (R1) failed to activate the promoter in either RCS cells or 10T1/2 cells. However, an element containing the 3′ repeat (R2) increased promoter activity in RCS cells in a copy-number-dependent manner, reaching several thousand-fold with 8 and 12 copies. Very little activation was detected in 10T1/2 cells with multiple copies of R2. Multiple copies of an 18-bp sequence containing the 3′ repeat therefore appeared sufficient to induce high levels of promoter activation in RCS cells.

Abolition of the activity of the 18-bp enhancer by specific mutations. To delineate the binding site for DNA-binding proteins potentially implicated in enhancer activity in RCS cells, constructions in which transversion mutations were introduced in the 18-bp R2 sequence or in a 20-bp R2* sequence, which contains two additional nucleotides at the 5′ end, were made (Fig. 2). Twelve copies of the R2 and R2* elements were similarly active in RCS cells (Fig. 2). Mutation of the nucleotide pair 5-6 slightly increased the activity of the enhancer in RCS cells, whereas mutation of the nucleotide pair 1-2, 8-9, 10-11, or 14-15 abolished activity. These results indicated that nucleotides both inside the repeat (8-9 and 10-11) and in the 5′ (1-2) and 3′ (14-15) flanking sequences were essential for enhancer activity. The importance of nucleotides located outside the repeat is in agreement with the absence of activity of R1, whose nucleotides flanking the repeat are different from those in R2.

Activity of *Col2a1* enhancer fragments in primary chondrocytes. The activities of *Col2a1* enhancer elements were tested in mouse rib chondrocytes by transfecting primary cells soon after their isolation from cartilage when they were still fully differentiated (14). The 89-bp *Col2a1* promoter was barely active in these cells but was strongly activated by *Col2a1* enhancer fragments that were active in RCS cells (Table 1). These included a two-copy 231-bp element, the 4-copy 48-bp A element, and the 12-copy 20-bp R2* element. Similar results were obtained with the 12-copy R2 and R2* elements (data not shown). The 231-bp element, previously shown to be a strong enhancer in RCS cells (17), contained the 156-bp enhancer plus 75 bp of the 5′ upstream sequence. Mutations in the 18-bp enhancer that abolished activity in RCS cells did the same in primary chondrocytes (Table 1). Hence, the minimal *Col2a1* enhancer elements were active in primary chondrocytes as well as in RCS cells.

TABLE 1. Transfection of primary chondrocytes with Col2a1 constructions

Intron segment[a]	Luciferase activity[b]
None	0.31 ± 0.16
2 × 231	271 ± 77
4 × A	39.5 ± 1.5
12 × R2*	153 ± 19
12 × m(1-2)R2*	0.98 ± 0.17
12 × m(14-15)R2*	0.50 ± 0.30

[a] The 89-bp *Col2a1* promoter construction (Fig. 1) was tested in parallel with constructions containing *Col2a1* intron 1 segment 2 × 231 (two tandem copies of a 231-bp enhancer segment spanning nucleotides +2113 to +2343 of the Col2a1 gene) or 4 × A (four copies of the A element [+2188 to +2234]) (Fig. 1) or one of the segments in Fig. 2.

[b] Average ± standard deviation for triplicate cultures of one representative experiment.

Activity of *Col2a1* enhancer fragments in transgenic mice. To verify the chondrocyte specificity of the minimal enhancer elements in vivo, we generated transgenic mice harboring four copies of A cloned in the vector used in our previous study with longer *Col2a1* enhancer fragments (28), in which a 309-bp *Col2a1* promoter drove the βgeo reporter gene (Fig. 3A). This promoter was unable by itself to direct cartilage expression (28). Two of three transgenic founder embryos collected 14.5 days postcoitus stained positively with X-Gal, a chromogenic substrate for β-galactosidase, whereas the third embryo showed no staining. The pattern of staining in the positive embryos was similar to the one obtained with longer enhancer fragments (28), although it was somewhat less intense. Whole-mount embryos showed staining in the cartilages of the ear and nose, in the cartilage anlagen of the limb long bones, in pelvic and shoulder girdles, and in vertebrae and ribs (Fig. 3B). Histological analysis of multiple sections throughout the whole embryos indicated that only chondrocytes stained with X-Gal (Fig. 3C). The A element thus appeared to be sufficient to confer chondrocyte-specific expression in vivo.

Of six transgenic mouse embryos shown by Southern analysis to harbor 12 copies of the R2 element cloned in the same vector, none stained with X-Gal (data not shown). Since the luciferase assay is more sensitive than X-Gal staining, transgenic mice were generated with the same construction that was used in transfections, i.e., with 12 copies of R2 cloned upstream of the 89-bp *Col2a1* promoter, itself linked to the luciferase gene. Significant luciferase activity was detected in newborn transgenic mice in extracts from rib cages and vertebrae which contained cartilage, from the brain and skin, and from the tail which contained both cartilage and skin besides other tissues (Table 2). All other nonchondrogenic organs were negative. Chondrocytes isolated from the ribs of transgenic mice contained high levels of luciferase activity (Table 2). Hence, although the construction allowed promiscuous expression in some nonchondrogenic tissues, our results indicated that the R2 element was able to direct promoter expression in chondrocytes in vivo.

FIG. 3. X-Gal staining of transgenic mice harboring four copies of element A. (A) Schematic representation of the DNA construction. Four tandem copies of the A element were inserted upstream of the SA-βgeo-bpA cassette and downstream of a *Col2a1* segment extending from −309 to +308. The SA-βgeo-bpA cassette includes a splice acceptor (SA), which allows correct splicing of the intron sequence; the βgeo gene, which encodes a fusion protein with *Escherichia coli* β-galactosidase and neomycin resistance activities; and the bovine growth hormone polyadenylation signal (bpA). The *Col2a1* segment contains 309 bp of promoter sequences, exon 1, and the proximal 70 bp of intron 1. The *Col2a1* translation initiation codon was mutated to CTG to favor translation initiation at the AUG codon of βgeo RNA. (B) Frontal and lateral views of two different transgenic founder embryos stained with X-Gal at 14.5 days postcoitus. (C) Histological analysis of a portion of a sagittal section of one of the two embryos shown in panel B. The section was counterstained with eosin. Bar, 400 μm.

TABLE 2. Luciferase activities in tissue extracts from transgenic mice harboring 12 copies of the R2 element[a]

Extract	Activity[b]		
	Mouse 1	Mouse 2	Mouse 3
Rib cage	173	27	19
Vertebrae	88	28	9
Brain	114	167	18
Skin	154	7	7
Tail	430	20	383
Calvarium			3
Muscle	7	1	2
Lung	3	0	0
Heart	5	0	1
Liver	1	0	0
Intestine	5	0	0
Spleen	1	0	5
Kidney	1	0	0
Thymus		2	2
Tail[c]	245	10	8
Chondrocytes[c,d]	1,478	250	392

[a] Transgenic mice were generated with a construction made of 12 copies of the R2 element cloned upstream of the 89-bp *Col2a1* promoter driving the luciferase gene (Fig. 1). Founder mice expressing the transgene were sacrificed within 3 days after birth, and luciferase activities in the indicated tissue extracts were measured.

[b] In relative luminescence units per microgram of protein.

[c] The last two rows of data correspond to three mice different from the three mice whose data are listed in the rest of the table.

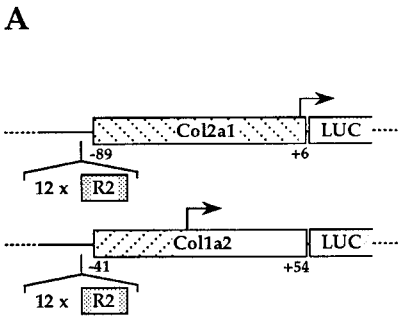
[d] Isolated from the rib cages of transgenic mice harboring the same construction as above. Luciferase activity was measured directly after digestion of rib cartilages by collagenase and extensive washes of the cells in phosphate-buffered saline.

Specific activation of a heterologous promoter by the 18-bp enhancer. We showed previously that the *Col2a1* promoter does not contain elements necessary for chondrocyte expression, using constructions in which a 231- or 182-bp *Col2a1* enhancer fragment was cloned upstream of either a minimal adenovirus major late promoter or a minimal β-globin promoter (17, 28). This result was confirmed with a construction containing the 12-copy R2 element cloned upstream of a minimal promoter (−41 to +54) of the mouse proα2(I) collagen gene (*Col1a2*) (Fig. 4). This short promoter, which contains no activating elements upstream of the TATA box (8), was essentially inactive in RCS and 10T1/2 cells, but it was significantly activated in RCS cells, not in fibroblasts, by the 12-copy 18-bp enhancer (Fig. 4). The level of activation of the minimal *Col2a1* promoter was, however, lower than that of the 89-bp *Col2a1* promoter. It is thus possible that the 89-bp *Col2a1* promoter contains elements not present in the shorter *Col2a1* promoter that support transactivation by the R2 multimer.

Proteins selectively expressed in RCS cells and primary chondrocytes bind a discrete sequence in the 18-bp enhancer. To determine whether RCS cells and chondrocytes express unique nuclear factors that specifically bind to the 18-bp enhancer and to locate the precise DNA binding sites of these factors, gel retardation assays were performed with nuclear extracts from various cell types and oligonucleotide probes containing the R2 element in its wild-type form and a series of mutant forms (Fig. 5A). These mutant probes each contained two different adjacent nucleotides modified by transversion.

Six major DNA-protein complexes were separated by electrophoresis after incubation of RCS cell nuclear extracts with the wild-type probe (Fig. 5B). Complexes 1, 2, 5, and 6 likely corresponded to ubiquitous proteins since they were formed with nuclear extracts from most cell types. Complex 3 was formed with nuclear extracts from RCS cells and was the major complex observed with extracts from primary chondrocytes. A complex with approximately similar mobility was also seen with nuclear extracts from the lymphoma EL-4 and Raji cell lines, but it was absent in nuclear extracts from fibroblast cell lines (10T1/2 and 714 cells), ROS osteosarcoma cells, and all other cell lines tested (C_2C_{12} myoblasts, HeLa cervical carcinoma cells, S194 myeloma cells, and NMuLi liver cells). Complex 4 was formed exclusively by nuclear extracts from RCS cells. We tentatively concluded that complex 3 contained one or several proteins selectively expressed in RCS cells and primary chondrocytes, whereas complex 4 contained one or several proteins present exclusively in RCS cells.

Oligonucleotide probes containing mutations in nucleotides 8 to 17 were unable to efficiently form complexes 3 and 4 or complex 1 (Fig. 5C). These included probes with mutations in the nucleotide pairs 8-9, 10-11, and 14-15 which were shown to abolish chondrocyte-specific enhancer activity completely (Fig. 2 and Table 1). In contrast, mutation of the nucleotide pair 1-2 which was also shown to abolish enhancer activity did not significantly affect the formation of any DNA-protein complex, nor did mutation of the nucleotide pair 3-4. Mutations of nucleotides 6 and 7 allowed stronger binding of proteins in complexes 3 and 4, and also complex 1, an effect which could be related to the slightly higher enhancer activity of the R2



B

Constructions	RCS	10T1/2
p89Col2a1	0.56	5.59
12 x R2 - p89Col2a1	1406	4.19
p41Col1a2	0.06	0.86
12 x R2 - p41Col1a2	18.8	0.64

FIG. 4. Activation of a heterologous promoter by 12 copies of the R2 element. RCS and 10T1/2 cells were transfected transiently with the following constructions. p89Col2a1 contained the 89-bp *Col2a1* promoter cloned upstream of the luciferase (LUC) gene in pLuc4 (Fig. 1); p41Col1a2 contained a 41-bp *Col2a1* promoter (−41 to +54) cloned upstream of the luciferase gene in pLuc4; 12xR2-p89Col2a1 and 12xR2-p41Col1a2 were made by cloning 12 copies of the R2 element directly upstream of the promoter in p89Col2a1 and p41Col1a2, respectively. Luciferase activities are averages for duplicate cultures in one representative experiment.

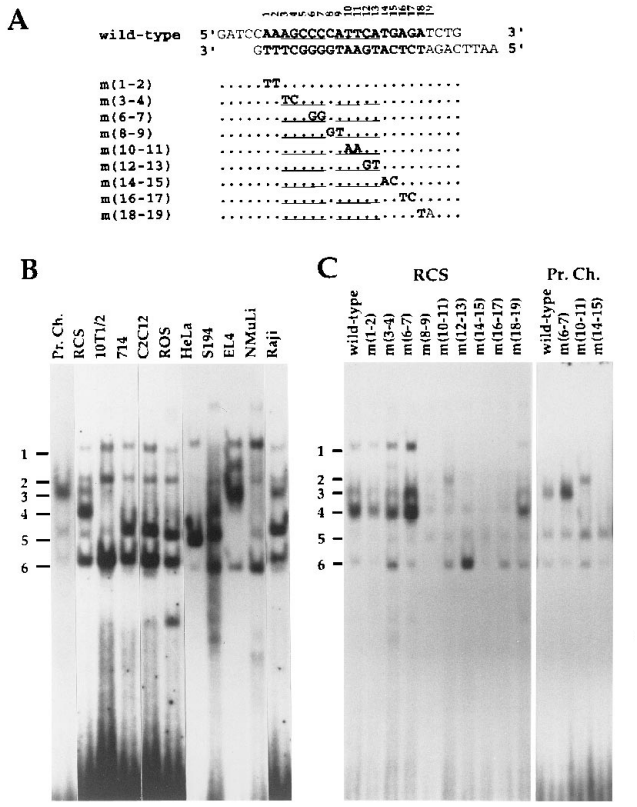


FIG. 5. Identification of chondrocyte-specific proteins binding to the R2 element. (A) Oligonucleotide probes used in gel retardation assays. The wild-type probe consisted of a double-stranded oligonucleotide corresponding to the 18-bp R2 element to which nucleotides were added to form a *Bam*HI restriction site at the 5′ end and a *Bgl*II restriction site followed by an *Eco*RI restriction site at the 3′ end. The nucleotides of the R2 sequence were numbered 1 to 18 from the 5′ to the 3′ side of the coding strand; number 19 was given to the proximal nucleotide of the 3′ flanking sequence. Mutant probes are designated by the letter m followed by the mutated nucleotides in parentheses. These probes were identical to the wild-type probe except for the mutated nucleotides. Only the coding strand is shown, with dots indicating nucleotides identical to those in the wild-type sequence. Nucleotides forming the 3′ repeat of element A are underlined. (B) Gel retardation assay with the R2 wild-type probe and nuclear extracts from various cell types. The following cell types were used: primary mouse rib chondrocytes (Pr. Ch.), RCS cells, 10T1/2 mouse embryo fibroblasts, subline 714 of BALB/3T3 mouse embryo fibroblasts, C_2C_{12} mouse skeletal myoblasts, ROS 17/2.8 rat osteosarcoma cells, HeLa human carcinoma epithelioid cells, S194 mouse myeloma cells, EL4 mouse lymphoma T-type cells, NMuLi mouse normal liver cells, and Raji human lymphoblast-like cells. Six major DNA-protein complexes, numbered 1 to 6, were separated by electrophoresis after incubation of the RCS cell nuclear extracts with the R2 probe. (C) Gel retardation assays with R2 mutant probes and nuclear extracts from RCS cells and primary chondrocytes. The major protein-DNA complexes formed by incubation of RCS cell nuclear extracts with the wild-type probe are indicated. Bands 2, 5, and 6 appear fainter in the RCS samples in panel C than in panel B because of the addition of 0.5 μg of poly(dG-dC) · poly(dG-dC) to the reaction mixtures. Note that bands 3 and 4 appear weaker with the m(1-2) probe than with the wild-type probe, but this was not the case in other experiments.

element that contained mutations in nucleotides 5 and 6. Finally, mutations of nucleotides 18 and 19 (nucleotide 19 is part of the flanking sequence added to the 18-bp element and does not correspond to the Col2a1 sequence) did not affect the formation of any DNA-protein complex. The proteins in complexes 1, 3, and 4 thus appeared to bind to the same sequence, CATTCATGAG, suggesting that these proteins might belong to the same family of DNA binding factors.

Only the 5′ part of this 10-bp binding site is conserved in the R1 element. In agreement with this partial homology, complexes 1, 3, and 4 were not observed when the R1 element was used as a probe in gel retardation experiments with nuclear extracts of RCS cells (data not shown). When the R1, R2, and mutant R2 oligonucleotides were used in competition with the R2 wild-type probe, results were consistent with those obtained in direct binding experiments, i.e., only the R1 oligonucleotide and the R2 oligonucleotides with mutations in nucleotides 8 to 17 were unable to compete for the formation of complexes 1, 3, and 4, whereas the R2 oligonucleotide competed efficiently (data not shown).

These results showed that proteins with a restricted pattern of cellular expression were present in RCS cells and primary chondrocytes and bound to the 18-bp enhancer. All mutations within the binding site for these proteins that were functionally tested in transfection experiments abolished enhancer activity, strongly suggesting a role of these proteins in enhancer activity. These results, however, do not explain why a mutation of the two nucleotides located at the 5′ end of the 18-bp enhancer abolished activity since no significant difference was seen in gel retardation assays whether a wild-type probe or a probe mutated in these two nucleotides was used. It is possible that these nucleotides were too close to the 5′ end of the oligonucleotide and therefore could not bind proteins efficiently. When larger oligonucleotides that extended more upstream were used, larger DNA-protein complexes were observed with extracts of RCS cells, but these complexes could not be distinguished from those obtained with extracts from 10T1/2 fibroblasts (data not shown).

The two chondrocyte-specific enhancer-binding proteins exhibit similar biochemical properties. In order to better characterize the chondrocyte-specific enhancer-binding proteins present in complexes 3 and 4, these proteins were extensively purified from RCS cell nuclear extracts by sequential chromotographies through two different DNA affinity columns, followed by Mono S and Mono Q ion-exchange columns (see Materials and Methods). The proteins present in these two complexes copurified through the four columns (data not shown). The Mono S fractions containing these proteins (Fig. 6A) were then fractionated by SDS-PAGE. Silver staining of the gel showed only a few protein species (Fig. 6B). The two most intense bands corresponded to proteins with apparent M_rs of 52,000 and 54,000. The relative intensity of these two bands and their Mono S elution profile were consistent with the hypothesis that the upper and lower bands in the SDS-PAGE corresponded, respectively, to complexes 3 and 4 in gel shift assays. DNA-binding experiments performed after elution-renaturation of proteins eluted from gel slices cut from another lane of the same gel as the one used for silver staining confirmed that the 54- and 52-kDa proteins formed, respectively, complexes 3 and 4 in gel shift assays (Fig. 6C and D). Gel retardation assays performed with purified proteins (Mono S eluates) and mutant R2 probes confirmed that the purified proteins were binding DNA to the 10-bp site identified with crude extracts (data not shown). Furthermore, methylation interference assay using purified proteins and a DNA probe containing the 48-bp A enhancer element also indicated that these proteins contacted DNA in the 10-bp site (data not shown).

These results indicated that the two chondrocyte-specific DNA-binding proteins have similar M_rs and must be closely related since they copurified and showed similar DNA-binding properties. Since each one of the two DNA-binding activities was recovered from gel slices that contained a single silver-stained band, it appears likely that the proteins bound DNA as monomers or as homodimers.

The chondrocyte-specific enhancer-binding proteins are likely POU domain proteins. A search in the Findpatterns database of the Genetics Computer Group (University of Wis-

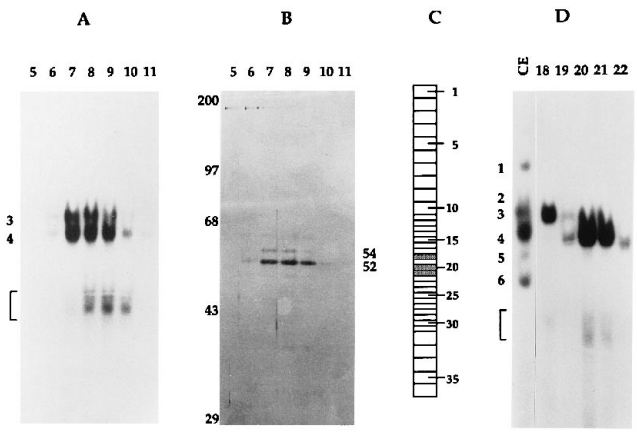


FIG. 6. Characterization of a purified preparation of chondrocyte-specific nuclear proteins. The cell-specific enhancer-binding proteins present in nuclear extracts from RCS cells were purified by chromatography through two different DNA affinity columns and then through Mono Q and Mono S columns, as described in Materials and Methods. The experiments in panels A to D were all performed with the same Mono S fractions. (A) Gel shift analysis. Mono S fractions were tested in gel retardation assay using the R2 probe. Only fractions 5 to 11 which contained the peak of activity of complexes 3 and 4 are shown. The micromolar salt concentrations of these fractions were as follows: 5, 122; 6, 147; 7, 176; 8, 202; 9, 233; 10, 252; 11, 275. A bracket indicates complexes of higher electrophoretic mobility which likely correspond to partially degraded purified proteins. No probe shift was detected in the flowthrough of the chromatography or in other elution fractions (data not shown). (B) Silver staining of proteins eluted in the Mono S fractions 5 to 11 and fractionated by SDS-PAGE. The M_rs of protein standards are indicated on the left, and the M_rs of two major protein species are indicated on the right. (C) Schematic representation of gel slicing. A pool of fractions 7 to 9 from Mono S chromatography was loaded on the same gel as in panel B. After electrophoresis and gel washes, the lane of the gel that contained the sample was cut into horizontal slices which were numbered as indicated. Shaded boxes represent gel slices 18, 20, and 21, from which proteins forming complexes 3 and 4 were recovered (see panel D). (D) Gel shift assay with proteins eluted from SDS-PAGE. Proteins eluted from the gel slices shown in panel C were tested in gel shift assay using the R2 probe. Only the assays for slices from which proteins forming complexes 3 and 4 were recovered are shown. Slice numbers are indicated at the top. Crude extracts (CE) from RCS cells were used as a standard, and complexes 1 to 6 formed with these extracts are indicated.

consin, Madison) indicated that the sequence of the 10-bp DNA-binding site, CATTCATGAG, might bind members of the POU domain protein family of transcription factors. This sequence contains a low-affinity heptamer consensus binding site (CTCATGA) on its lower strand and an overlapping imperfect high-affinity octamer binding site for these proteins on its upper strand (ATTAATGC). A characteristic of POU domain proteins is their flexibility in DNA sequence recognition (9). We, therefore, investigated whether the chondrocyte-specific DNA-binding proteins might be POU domain proteins.

We performed gel shift assays using in parallel the wild-type R2 enhancer probe (R2) and a probe (OCT) containing a consensus octamer binding site for POU domain proteins. Three major DNA-protein complexes, 1*, 3*, and 4*, were obtained after incubation of RCS cell nuclear extracts with the OCT probe (Fig. 7A, lanes 11 and 16). The electrophoretic mobilities of these complexes were similar to those of complexes 1, 3, and 4 formed with the R2 probe (lanes 1 and 6). The OCT oligonucleotide competed very efficiently for the formation of complexes 1, 3, and 4 with labeled R2, at least 10 times better than unlabeled R2 itself (compare lanes 1 to 5 with lanes 6 to 10); accordingly, R2 competed less efficiently for the binding of proteins to the OCT probe than unlabeled OCT (compare lanes 11 to 15 with lanes 16 to 20). These results indicated that the proteins forming complexes 1, 3, and 4 with R2 were likely the same as those forming complexes 1*, 3*, and 4*, respectively, with OCT. Furthermore, these proteins likely belonged to the POU domain protein family since they bound with a higher affinity to the OCT oligonucleotide that contains a strong binding site for POU domain proteins than to the low-affinity and imperfect binding sites for these proteins in R2. Nuclear extracts from primary chondrocytes formed two major complexes with OCT, which corresponded to complexes 1* and 3* from RCS cell nuclear extracts (data not shown). Complex 3* was much more abundant than complex 1*, and, similarly, complex 3 formed with R2 was much more abundant than complex 1. These results further suggest that the specific proteins identified in nuclear extracts from RCS cells and primary chondrocytes were identical. The specific proteins purified from RCS cells also bound very efficiently to OCT (Fig. 7B), confirming that the proteins that bound to the two probes were identical.

Since complex 1 was formed with a protein widely expressed (Fig. 7A) and since Oct-1 is a ubiquitous POU domain protein, we asked whether complex 1 contained Oct-1. An antiserum containing antibodies against Oct-1 produced a supershift of complexes 1 and 1* formed with nuclear extracts from RCS cells (Fig. 7C) and from other cell types that were tested (data not shown), with both the R2 probe and the OCT probe, thus confirming that complex 1 contained Oct-1.

Antibodies against the predominantly lymphoid-restricted Oct-2 appeared to supershift the complex formed with EL-4 and Raji nuclear extracts that had approximately the same mobility as complexes 3 and 3*, but these antibodies did not supershift complex 3* or complex 4*, formed either with RCS cell nuclear extracts (Fig. 7D) or with purified proteins from RCS cell extracts (data not shown). These results strongly suggested that the protein present in complex 3* formed with chondrocyte extracts very likely corresponds to another POU domain protein.

Altogether, our DNA binding experiment data strongly suggest that RCS cells and primary chondrocytes express one or two members of the POU domain protein family which bind to a 10-bp sequence in the minimal *Col2a1* enhancer element. We hypothesize that these proteins play a role in enhancer activity.

Abolition of the activity of 465- and 231-bp enhancer elements by deletion of the 10-bp binding site for chondrocyte-specific proteins. In order to determine whether the 10-bp sequence which binds chondrocyte-specific proteins and is essential for the enhancer activity of the multimerized 18-bp sequence in chondrocytes was also essential for the activity of larger intron 1 fragments (17, 28), we tested the activities of enhancer segments of 465 and 231 bp in which these 10 bp were deleted (Fig. 8). Whereas the 465-bp wild-type segment present as a single copy stimulated promoter activity in RCS cells, the (465 − 10)-bp element was inactive (Fig. 8A). Similarly, the 231-bp wild-type fragment stimulated promoter activity in RCS cells when tested as one copy and about 10 times more than that when tested as two tandem copies, but constructions containing either one copy or two copies of the (231 − 10)-bp fragment showed no significant enhancer activity in RCS cells (Fig. 8B). The 465-bp fragment was inactive in 10T1/2 fibroblasts, but, interestingly, the (465 − 10)-bp fragment slightly increased promoter activity in these cells (Fig. 8A). The wild-type 231-bp segment modestly increased promoter activity in 10T1/2 cells, but a similar level of activation was obtained with one and two copies. The 10-bp deletion did not affect or slightly stimulated the promoter activation induced by, respectively, one copy or two copies of the 231-bp segment (Fig. 8B). Although the higher level of promoter activation obtained in fibroblasts with constructions containing the 10-bp deletion was reproducible (data not shown), its sig-

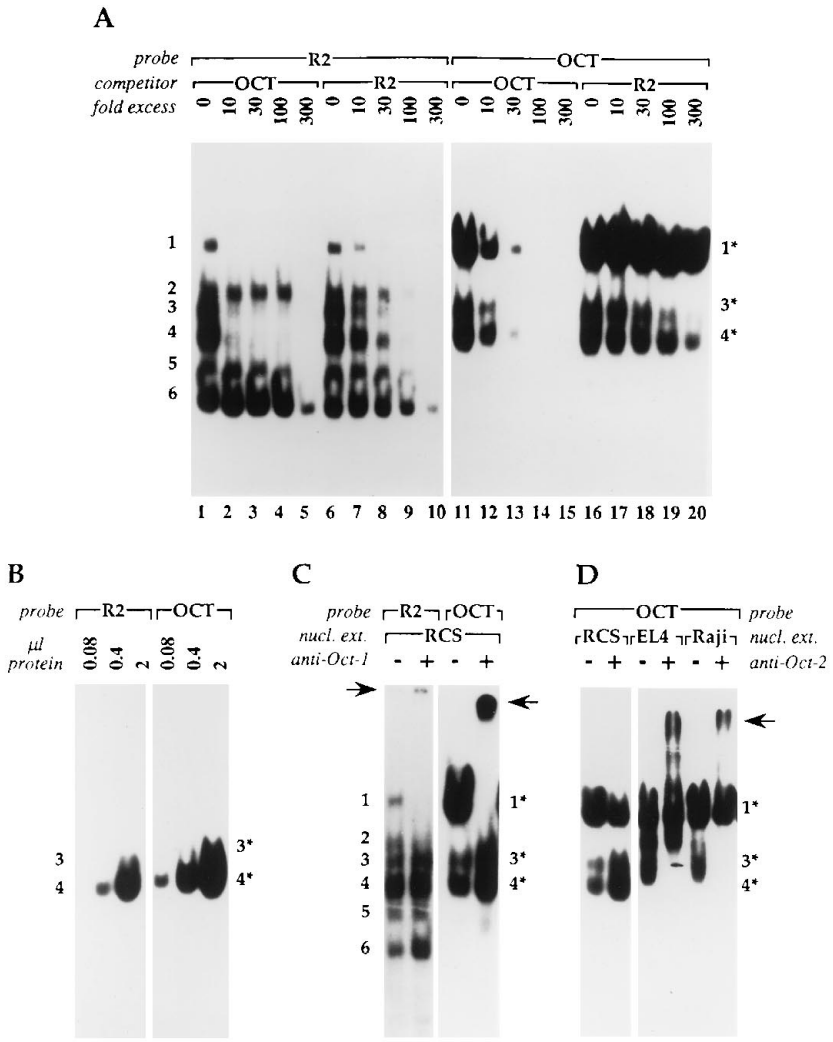
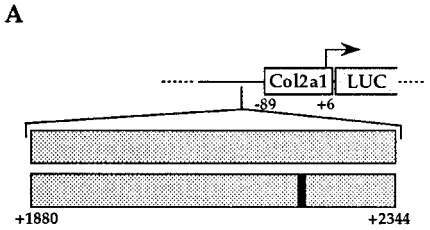


FIG. 7. Gel shift analyses using the R2 and OCT probes and antibodies against Oct-1 and Oct-2. (A) Direct binding and competition assays. Nuclear extracts from RCS cells were incubated with the R2 or OCT probe and with a 0- to 300-fold excess of an unlabeled competitor probe, as indicated. The major complexes obtained with the R2 probe are labeled 1 to 6, and the major complexes obtained with the OCT probe are labeled 1*, 3*, and 4*. (B) DNA-binding assays with purified proteins. Increasing amounts of a purified preparation of RCS cell-specific nuclear proteins (0.08, 0.4, and 2 μl) were incubated with the R2 or OCT probe. Note that the preparation used in this experiment was enriched in protein forming complex 4 relative to protein forming complex 3; in other experiments, the purified protein forming complex 3 with the R2 probe also bound tightly to the OCT probe. (C) Supershift of complexes 1 and 1* with antibodies directed against Oct-1. RCS cell nuclear extracts (nucl. ext.) were incubated with the R2 or OCT probe, in the presence (+) or absence (−) of 2 μl of antiserum containing antibodies against Oct-1. Supershifts are indicated with arrows. No supershift was observed with a nonimmune serum (data not shown). (D) Supershift of Oct-2 with specific antibodies. Nuclear extracts from RCS, EL-4, and Raji cells were incubated with the OCT probe in the presence (+) or absence (−) of antibodies directed against Oct-2. Supershifts are indicated with an arrow.

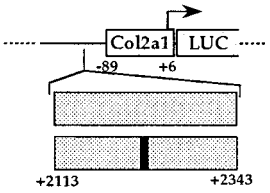
nificance is not understood. It is possible that the deletion created a binding site for transcriptional activators present in fibroblasts that would otherwise never be allowed to play a role in the context of a wild-type enhancer. Alternatively, it is possible that fibroblasts contain proteins that bind to the wild-type enhancer, not the deleted version, and that decrease the low degree of activity of the chondrocyte enhancer in fibroblasts. These results nevertheless indicated that the 10-bp binding site for chondrocyte-specific proteins was involved in the high-level chondrocyte-specific activity of the *Col2a1* intron 1 enhancer.

DISCUSSION

We have delineated a minimal 18-bp sequence in the first intron of the Col2a1 gene which after multimerization enhanced promoter activity several thousand-fold in RCS cells and primary chondrocytes and only minimally in fibroblasts. This multimerized enhancer also generated promoter expression in chondrocytes of transgenic mice. The 18-bp sequence is part of a DNA motif which is highly conserved between the human, rat, and mouse genes and which is made up of two inverted repeats of 11 bp each, separated by an 18-bp linker. The repeats themselves contain no consensus binding site for known DNA-binding proteins, and their significance is not yet understood. The 18-bp enhancer includes the 3′ repeat and flanking nucleotides. Mutational analysis has shown that nucleotides both inside the repeat and in the 5′ and 3′ flanking sequences were essential for enhancer activity. Interestingly, an element that included the 5′ repeat and flanking nucleotides was inactive. This result further emphasized the role in enhancer activity of nucleotides adjacent to the 3′ repeat since the nucleotides flanking the two repeats are different.



Intron 1 Segments	RCS	10T1/2
none	0.01	0.09
465 bp	0.56	0.03
(465-10) bp	0.04	0.20



Intron 1 Segments	RCS	10T1/2
none	0.01	0.13
1 x 231 bp	2.17	0.78
1 x (231-10) bp	0.03	0.64
2 x 231 bp	20.7	0.86
2 x (231-10) bp	0.08	1.67

FIG. 8. Effect of a 10-bp deletion in large intron 1 fragments on enhancer activity. (A) Deletion within a 465-bp intron 1 segment. DNA constructions were made similarly to those in Fig. 1 by using as a tester enhancer either a 465-bp intron 1 fragment (spanning nucleotides +1880 to +2344 in the Col2a1 gene) or a corresponding intron 1 segment [(465-10) bp] in which the 10-bp binding site for chondrocyte-specific proteins had been deleted (nucleotides +2224 to +2233 [solid portion]). These constructions and a construction containing the promoter only were transfected transiently in RCS and 10T1/2 cells. Luciferase activities are shown as averages for duplicate cultures in one representative experiment. (B) Deletion in a 231-bp intron 1 segment. Constructions similar to those used in panel A were made by using either a 231-bp intron 1 enhancer fragment (+2113 to +2343) or a corresponding intron 1 segment containing the same 10-bp deletion as in the 465-bp segment. These segments were cloned as one copy (1×) or as two tandem copies (2×), and the constructions were transfected transiently in RCS and 10T1/2 cells. Luciferase activities are averages for duplicate cultures of one representative experiment.

Gel retardation assays have indicated that nuclear proteins selectively expressed in RCS cells and primary chondrocytes bind to a 10-bp sequence located at the 3′ end of the 18-bp enhancer. Point mutations within this site both abolished the binding of these proteins and blocked the activity of the multimerized 18-bp enhancer. Moreover, deletion of these 10 bp in intron 1 segments of 465 and 231 bp resulted in essentially complete loss of enhancer activity in RCS cells. These results therefore indicated that this 10-bp *cis*-acting element plays a central role in the activity of the chondrocyte enhancer and strongly suggest that the chondrocyte-specific proteins are key players in the activation of the Col2a1 gene in chondrocytes.

One of the chondrocyte-specific proteins was present exclusively in RCS cell nuclear extracts. The other one was present both in RCS cells and in primary chondrocytes but in no other cell type tested. A computer search indicated that the sequence of the 10-bp protein binding site contains a low-affinity heptamer binding site for members of the POU domain family of transcription factors and might also contain an overlapping imperfect high-affinity octamer binding site for these factors. Several lines of evidence that the chondrocyte-specific proteins might indeed belong to this family were obtained. The proteins bound with higher affinity to a probe containing a consensus octamer binding site for POU domain proteins than to the 18-bp Col2a1 probe. Antibodies directed against Oct-1, a ubiquitous POU domain protein, supershifted a complex that was formed with all nuclear extracts tested and that presented DNA binding properties similar to those of the chondrocyte-specific proteins. Elution-renaturation experiments indicated that the chondrocyte-specific proteins bound DNA either as monomers or as homodimers, a result in agreement with the ability of POU domain proteins to bind DNA as monomers. Since no POU domain protein is known to be expressed specifically in cartilages, one can speculate that chondrocytes express still unknown members of this family. Although Oct-1 is ubiquitously expressed and involved in the expression of housekeeping and cell-specific genes, several other POU domain proteins show a cell-type-restricted pattern of expression and have been implicated in tissue-specific expression of various genes (9). Analytical purification indicated that the two chondrocyte proteins were closely related since they copurified through several DNA affinity and ion-exchange chromatographies. Moreover, their apparent M_rs differ by only 2,000. It is possible therefore that they represent two different products of one gene. Large-scale purification of the proteins will be necessary to obtain partial amino acid sequences, knowledge of which would then be used to isolate cDNAs.

Our mutational and deletion analyses also indicated that besides the 10-bp binding site for chondrocyte-specific proteins multiple other sequences present in *Col2a1* intron 1 and the promoter were important for enhancer activity. These other elements might bind proteins that cooperate with each other and with the chondrocyte-specific proteins in order to achieve high-level promoter activation. One such element must exist at the 5′ end of the 18-bp enhancer since a mutation of the two most-5′ nucleotides abolished enhancer activity. Other elements are probably present in the 48-bp element outside the 18-bp sequence since four copies of the 48-bp element were able to enhance promoter activity in chondrocytes at a much higher level than four copies of the 18-bp element. We have also demonstrated that the 3′ part of the 156-bp intron 1 enhancer fragment harbored two sequences each of which potentiated the activity of the 48-bp enhancer. We have shown previously that these sequences were similarly footprinted by nuclear extracts from 10T1/2 fibroblasts and RCS cells, suggesting that they might bind ubiquitous proteins (17). Along

with the progressive truncation of *Col2a1* intron 1 performed to delineate elements involved in the chondrocyte specificity of the enhancer, it appeared that more and more copies of active fragments were gradually necessary to generate promoter activation. Multimerization of short elements might compensate for the progressive deletion of elements important for enhancer strength in much larger DNA segments. Cooperation between enhancer-binding proteins and proteins binding to the 89-bp *Col2a1* promoter could also be implicated in enhancer strength since the level of promoter activation obtained with a minimal *Col2a1* promoter was much lower than with the 89-bp *Col2a1* promoter. It can be mentioned here that promoter activation was specific to RCS cells regardless of whether a *Col2a1* or *Col2a1* promoter was used, confirming our previous observation that the *Col2a1* promoter is not required for chondrocyte expression (17, 28). In agreement with our results, Savagner and collaborators recently showed that elements located within the proximal promoter of the *Col2a1* gene were required for high-level expression of constructions containing a *Col2a1* intron 1 enhancer segment in chicken embryo chondrocytes (20).

In transgenic mice, four copies of the 48-bp enhancer conferred high levels of chondrocyte expression on a *lacZ* reporter gene. Twelve copies of the 18-bp enhancer cloned in the same vector could not activate this promoter to levels sufficient to detect staining of chondrocytes by X-Gal. However, when 12 copies of the 18-bp enhancer were cloned upstream of a promoter driving the luciferase gene, promoter activity was detected by the very sensitive luciferase assay in cartilage tissues and chondrocytes isolated from newborn mice and not in most other tissues. Since 4 copies of the 48-bp element were less active than 12 copies of the 18-bp sequence, it is possible that the 48-bp enhancer contains DNA elements which bind a factor(s) active in opening the chromatin in vivo, eventually in conjunction with chondrocyte-specific protein(s) binding to the 18-bp element, and that these elements would have been deleted when the enhancer was shortened to 18 bp.

In addition to expression in cartilages, the construction containing 12 copies of the 18-bp enhancer cloned upstream of the 89-bp *Col2a1* promoter was also active in the skin and brain in newborn transgenic mice. Similarly, we had previously shown that some expression in the brain was present in addition to cartilage expression in transgenic mice harboring a 182-bp intron 1 fragment cloned upstream of a minimal β-globin promoter (28). We do not know whether this construction was expressed in the skin since transgenic embryos were stained with X-Gal at 14.5 days of development, when the skin had not formed yet. Since we observed a perfect cartilage-specific pattern with four copies of the 48-bp segment linked to a 309-bp *Col2a1* promoter, it is likely that elements which inhibit expression in the brain, and possibly also in the skin, are located in the *Col2a1* promoter between −309 and −89 or in the 48-bp element outside the 18-bp enhancer. It is possible that the expression in brain and skin tissues corresponds to the low-level expression of Col2a1 revealed in these tissues by in situ hybridization (2). Although we have not tested the 89-bp *Col2a1* promoter by itself in transgenic mice, it is unlikely that the cartilage expression observed with the construction containing the 18-bp enhancer and the 89-bp *Col2a1* promoter was generated by the promoter itself since a 309-bp *Col2a1* promoter was unable by itself to confer cartilage expression in transgenic mice (28).

While the manuscript was being completed, a 100-bp segment of the rat *Col2a1* intron 1 was reported as having the minimum size necessary for chondrocyte-specific expression in DNA transfection experiments (13). This element extends 41 bp upstream and 12 bp downstream of the 48-bp enhancer segment described here. One substitution mutation and two internal deletions in a region corresponding to our 18-bp enhancer greatly decreased activity, whereas internal deletions in an AT-rich region in the 5′ portion of this 100-bp element reduced activity by about one-half. We hypothesize that the AT-rich sequence can potentiate the promoter activation generated by the 18-bp chondrocyte enhancer.

In conclusion, we have identified an 18-bp segment in the first intron of the mouse Col2a1 gene that was sufficient to direct promoter expression in chondrocytes of transgenic mice and in transiently transfected RCS cells and primary chondrocytes. Mutation in a 10-bp sequence within this segment or deletion of these 10 bp prevented enhancer activity and also abolished the binding of proteins specifically expressed in RCS cells and chondrocytes. These proteins likely belong to the POU domain protein family. We speculate that they are key factors in the activation of the Col2a1 gene in chondrocytes and might also be involved in the expression of other genes selectively activated during chondrocyte differentiation. Our data also pointed out other elements located in the first intron and in the promoter of the Col2a1 gene which likely bind proteins that help generate the high level of expression of the Col2a1 gene in chondrocytes.

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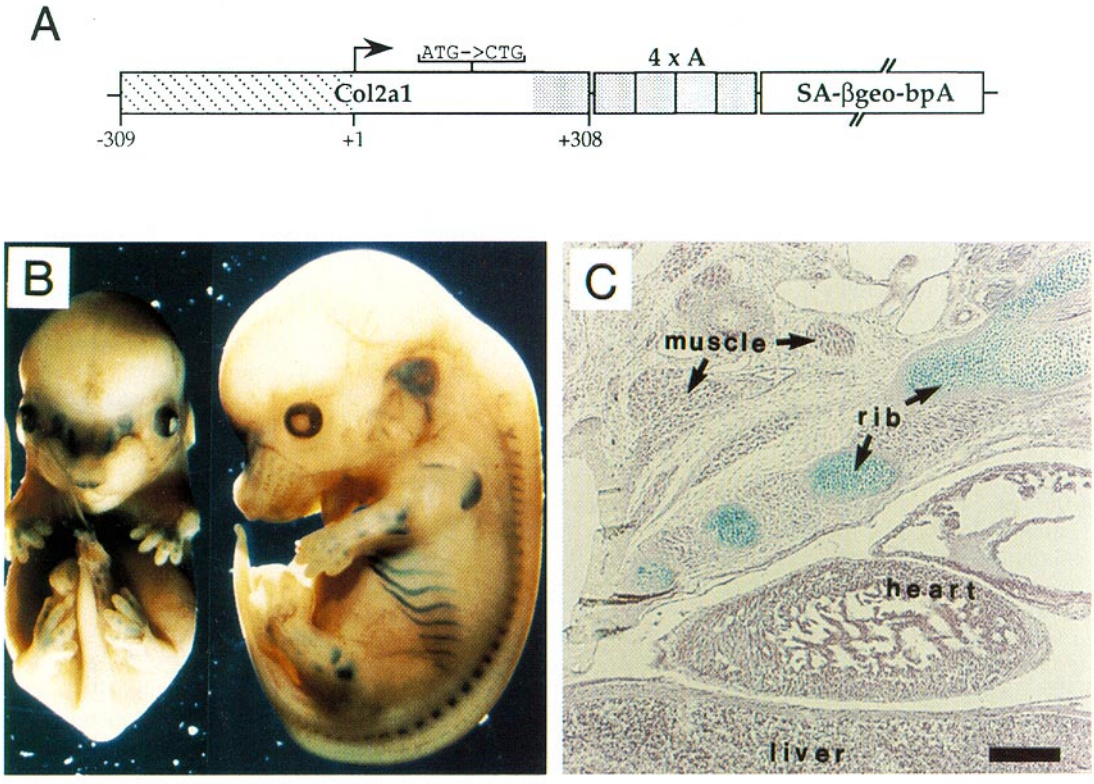
A
ATG->CTG
4 x A
Col2a1
SA-βgeo-bpA
-309
+1
+308
B
C
muscle
rib
heart
liver