Identification of an Enhancer Sequence within the First Intron Required for Cartilage-specific Transcription of the α2(XI) Collagen Gene*

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Type XI collagen, a heterotrimer composed of α1(XI), α2(XI) and α3(XI), is primarily synthesized by chondrocytes in cartilage and is also present in some other tissues. Type XI collagen plays a critical role in collagen fibril formation and skeletal morphogenesis. We investigated a tissue-specific transcriptional enhancer in the first intron of the α2(XI) collagen gene (Col11a2). Transient transfection assays using reporter gene constructs revealed that a 60-base pair (bp) segment within intron 1 increased promoter activity of Col11a2 in rat chondrosarcoma cells but not in either Balb/c3T3 cells or undifferentiated ATDC5 cells, suggesting that it contained cell type-specific enhancer activity. In transgenic mice, this 60-bp fragment was also able to target β-galactosidase expression to cartilage including the limbs and axial skeleton, with similar localization specificity as the full-length intron 1 fragment. Competition experiments in gel shift assays using mutated oligonucleotides showed that recombinant Sox9 bound to a 7-bp sequence, CTCAAAG, within the 60-bp segment. Anti-Sox9 antibodies supershifted the complex of the 60-bp segment with recombinant Sox9 or with rat chondrosarcoma cell extracts, confirming the binding of Sox9 to the enhancer. Moreover, a site-specific mutation within the 7-bp segment resulted in essentially complete loss of the enhancer activity in chondrosarcoma cells and transgenic mice. These results suggest that the 7-bp sequence within intron 1 plays a critical role in the cartilage-specific enhancer activity of Col11a2 through Sox9-mediated transcriptional activation.

Cartilage is a highly specialized tissue important for bearing compression loads in joints and also serves as the template for most developing bones. Cartilage contains unique extracellular matrix proteins produced by chondrocytes. The collagen network provides the scaffolding of the cartilage matrix and confers tensile strength important for resisting compression in cartilage. Collagen fibrils in cartilage consist of the major collagen, type II, and minor collagens, type IX and type XI. Type XI collagen, consisting of α1(XI), α2(XI), and α3(XI) chains, co-assembles with type II collagen to form cartilage collagen fibrils, whereas type IX collagen is associated with the surface of these fibrils (1, 2). The α1(XI) and α2(XI) chains are distinct gene products (3, 4), whereas the α3(XI) chain is a post-translational variant of the α1(II) chain (5). It has been postulated that type IX and type XI collagens regulate the collagen network by determining the diameter of cartilage collagen fibrils and their interactions with other matrix components (6–9).

Mutations in the type XI collagen genes have been identified and found to cause chondrodysplasia in both humans and mice. For example, mutations in the α2(XI) collagen gene (Col11a2) were identified in patients with Stickler syndrome and otophysyseal dysplasia (10). A null mutation in the α1(XI) gene of cho (chondrodysplasia) mice causes dwarfism with reduced matrix and thickened cartilage collagen fibrils (11). These findings indicate that type XI collagen plays an important role in skeletal morphogenesis and that expression of type XI collagen is critical for the development of cartilage.

Several regulatory regions of the mouse α2(XI) collagen gene have been identified, and their modular arrangements have been proposed (12–14). The −742 promoter segment is able to direct transcription of Col11a2 in most cartilaginous tissues in transgenic mice (15–17) and consists of at least two chondrocyte-specific enhancers containing Sox9 binding sites at −600 and −530 (15–19). Inclusion of a 2.3-kb segment from the first intron to the −742 promoter construct increased the promoter activity of Col11a2 (15). A shorter promoter construct (−453) did not show cartilage-specific expression; however, inclusion of the intron sequence to the construct induced reporter gene expression in cartilage. These results suggest the presence of the enhancer in intron 1. More recently, it was found that the −530 promoter sequence was sufficient for cartilage-specific expression of Col11a2 and that deletion of a sequence between −530 and −500 abolished reporter gene expression in cartilage, suggesting the presence of regulatory elements within this region required for cartilage-specific expression (13, 16).

SOX9 was initially identified by positional cloning as associated with the skeletal malformation syndrome campomelic dysplasia (20). It contains an HMG-type DNA-binding domain and a transactivation domain (21). The HMG box region of SOX9 binds DNA at the target sequences AACAT and AA-CAGA (22). SOX9 is expressed in primordial cartilage and in other noncartilaginous tissues during development (19, 23). It has been shown that SOX-9 regulates Col2a1 transcription through Sox9 binding to the intron enhancer (18, 24, 33). It has also been shown that the promoter of Col11a2 contains Sox9-binding sites necessary for cartilage-specific expression (16).

In this study, we have characterized the activity of the intron enhancer of Col11a2 by deletion analysis in cell cultures and in

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1 The abbreviations used are: kb, kilobase(s); HMG, high mobility group; bp, base pair(s); RCS, rat chondrosarcoma; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay.
transgenic mice and by DNA binding assays. We found that a 60-bp sequence from intron 1 can direct cartilage-specific expression of Col11a2. Mutation analysis identified a 7-bp sequence within the sequence critical for cell type-specific enhancer activity and Sox9 interactions.

MATERIALS AND METHODS

Cell Cultures—The rat chondrosarcoma RCS cell line was kindly provided by Dr. James Kimura (Henry Ford Hospital, Detroit, MI). RCS cells synthesize matrix proteins characteristic of chondrocytes and have been used for many years as a cell line for the study of cartilage development. The RCS cell line ATDC5 was kindly provided by Dr. Yuji Hiraki (Kyoto University, Kyoto, Japan). The RCS and BALB/3T3 cell lines were maintained in Dulbecco’s modified Eagle’s medium:high glucose, without pyruvate (Life Technologies, Inc.) (25). The ATDC5 cell line was cultured in Dulbecco’s modified Eagle’s medium:nutrient mixture Ham’s F-12 medium (1:1) (Life Technologies, Inc.) (26). All cell media were supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% (v/v) FCS or 5% (v/v) ATDC5 cells) heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) or 10% heat-inactivated calf serum (BALB/3T3 cells) (Hyclone Laboratories, Inc.). All cultures were fed with fresh medium every 2–3 days and cultured at 37 °C under 10% CO₂.

Transient DNA Transfections—Transfections of plasmid DNA into RCS cells, BALB/3T3 cells, and undifferentiated ATDC5 cells were performed using the Fugene transfection kit (Roche Molecular Biochemicals). Luciferase reporter plasmids were cotransfected with the pRLSV40 plasmid (27) as an internal control for transfection efficiency. Luciferase activities were assayed by the Dual-Luciferase™ Reporter Assay System (Promega, Madison, WI). Relative luciferase activities were expressed as ratios of luciferase activities of the experimental vectors to the internal control vector.

Construction of Col11a2-Luciferase Reporter Genes—All Col11a2 reporter gene constructs were derived from the plgel3 vector (Promega), which contains a poly(A) signal, the luciferase reporter gene, and a SV40 late poly(A) signal. Constructs 453Luc, 530Luc, and 453Luc contain a 1122-bp fragment (−742 to +380) of the α2XI gene, a 910-bp fragment (−530 to +380), and an 833-bp fragment (−453 to +380), respectively, cloned into the EcoRI and XhoI sites of pGL3. 453Luc, 530Luc, and 453Luc were digested with BglII and HpaI to prepare a DNA fragment free from the vector sequence. The plasmids were transfected into RCS cells, which express type XI collagen, and into BALB/3T3 and undifferentiated ATDC5 cells. The cell extracts (50 μg) were separated by 4–20% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated with the antibodies to Sox9 (1:500). Antibody-reacted material was detected with a horseradish peroxidase-conjugated secondary antibody and visualized with ECL. Double-stranded oligonucleotide probes, HMG, and 60-bp oligonucleotide consisted of the following sequences: HMG (5′-ggGACT-GAGAACAACGGGGCTCGAGCAG) (22) and 60-bp oligonucleotide 5′-ggGCGGTTTCTCTACGCTGTAA (23) were translated in vitro. Transgenic mice were produced by microinjecting the DNA fragment into the pronuclei of fertilized eggs from female mice (FVB/N) as described (28). Transgenic founder mice were sacrificed at 14.5 days postcoitus. PCR analysis for genotyping and staining for β-galactosidase was performed as described previously (15). Three embryos out of 24 were transgenic and showed similar β-galactosidase staining patterns.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from RCS cells as described (29). A full-length cDNA for Sox9 (GenBank™ accession number R47011) was identified through randomly selecting over 1000 cDNA clones from a rat incisor cDNA library and partially sequencing them in the Oral and Craniofacial Genome Anatomy Project (35). The full coding sequence of the Sox9 cDNA was cloned into the pCITE-4a in vitro translation vector (Noven), and recombinant Sox9 was synthesized in vitro using TNT Coupled Reticulocyte Lysate Systems (Promega). Sox9 antibodies were generated by immunizing rabbits with recombinant Sox9 synthesized in bacteria using the PQE expression vector (Qiagen), and IgG fractions of the antiserum were used for supershift experiments in EMSA. Western blots with antibodies to Sox9 were performed using cell extracts from RCS, ATDC5, and BALB/3T3 cells. The cell extracts (50 μg) were translated in vitro and incubated with 32P-dCTP and a large double-stranded fragment of DNA from the rat Sox9 gene. EMSA was performed as described (30). Briefly, nuclear extract or in vitro translated Sox9 was incubated for 30 min at room temperature in mobility shift buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM CaCl₂, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) and 2 μg of poly(dI-dC) with 30,000 cpm of 32P-end-labeled, double-stranded probe in a 30-μl volume. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel containing 4.5 mM Tris-HCl, pH 7.5, 4.5 mM boric acid, and 1 mM EDTA. The gels were dried, exposed, and visualized by autoradiography. For competition analysis, 100-fold excess unlabeled oligonucleotides were included in the initial incubation.

RESULTS

Cell Type-specific Enhancer Activity of the Col11a2 First Intron Sequence in RCS Cells—Using transgenic mice, we previously identified several regulatory regions of the Col11a2 gene important for cartilage-specific transcription (15). These studies located an enhancer within the first intron of Col11a2. Here we have examined this activity by transient transfection assays in cell cultures to further delineate and characterize the enhancer of intron 1. Several luciferase reporter gene constructs with the Col11a2 promoter and intron sequence were prepared and transfected into RCS cells, which express type XI collagen, and into BALB/3T3 and undifferentiated ATDC5 cells, which do not express type XI collagen (Fig. 1). The −742 promoter segment (742Luc) showed the highest activity in RCS cells, which was more than 10-fold greater than that observed in the two other cell types. The −530 promoter segment (530Luc) was less active compared with 742Luc (about 60% reduced). Low activity of the 530 promoter segment (530Luc) was observed in all three cell types. The higher activity of 742Luc over that of 530Luc is due to the presence of two enhancers at −600 and −520 in the promoter region of Col11a2, whereas 530Luc contains only one enhancer (16). These results agree with those obtained in transgenic mice (13) and in cell cultures (16), suggesting the presence of at least two enhancers in the promoter region, between −742 and −530 and between −530 and −453.
Inclusion of a 2.3-kb intron segment of Col11a2 to these promoter constructs increased promoter activity in RCS cells (Fig. 1). The −453 promoter construct showed little activity. However, there was a significant increase when the 2.3-kb segment was included to the −2453 promoter segment. All constructs showed little activity in either Balb3T3 or ATDC5 cells. The level of activity in these cell types is not due to poor transfection efficiency because transfection efficiency was normalized using an internal control construct. About 25 and 80% increases were observed when the intron segment was included in the −2742 and −2530 promoters, respectively. These results are in agreement with the previous observation in transgenic mice and suggest that the 2.3-kb intron 1 segment contains an enhancer for cell type-specific expression.

Deletion Analysis of the Intron 1 Enhancer—We next determined the minimum size of the intron 1 enhancer required for cell type-specific expression by examining the activities of intron 1 deletion mutant constructs with the −453 promoter segment in RCS cells (Fig. 2). 453Luc/Int4 containing a +651 to +1460 segment from deletion of the 2.3-kb intron 1 retained full enhancer activity. When a 300-bp region (+1161 to +1460) was deleted (453Luc/Int5) from the 453Luc/Int4 construct, a 91% decrease in activity was observed, suggesting that the 300-bp segment has enhancer activity. This was confirmed by the observation that the 300-bp segment alone (453Luc/Int8) has 84% activity of 453Luc/Int4. None of the constructs showed significant activity in either BALB3T3 or undifferentiated ATDC5 cells. Thus, these results suggest that the 300-bp region (+1161 to +1460) within intron 1 contains a regulatory element required for expression of Col11a2 in RCS cells in the absence of the upstream Sox9-containing enhancer elements.

Identification of an Enhancer in the 300-bp Segment of Intron 1—To further localize the enhancer activity in the 300-bp segment, we prepared a series of 30-bp double-stranded oligonucleotides, which covered the whole segment, and used them as competitors in transfection assays (Fig. 3). In the competition assay, the 453Luc/Int8 reporter gene construct was transfected into RCS cells with 100-fold molar excess of the various 30-bp double-stranded oligonucleotides (Int8-1 to Int8-10). The enhancer activity of 453Luc/Int8 was competed by oligonucleotides Int8-6 and Int8-7 but not by the other oligonucleotides. These results suggest that the 60-bp sequence (+1311 to +1370) corresponding to oligonucleotides Int8-6 and Int8-7 may contain the enhancer activity.

Activity of the 60-bp Enhancer of Intron 1 Col11a2 in RCS Cells—We next examined the enhancer activity of the 60-bp segment in RCS cells by transfection assays. We prepared 453Luc constructs containing various copies of the 60-bp sequence and tested their enhancer activity (Table I). A single copy of the 60-bp construct (453Luc/1 × 60) increased the promoter activity 10-fold compared with 453Luc without the fragment. Increasing copy numbers progressively increased the promoter activity up to 150-fold with eight 60-bp copies (453Luc/8 × 60). This indicated that the 60-bp segment was
able to enhance promoter activity of Col11a2. These results confirm that the 60-bp segment contains enhancer activity in RCS cells.

**Specific Protein and DNA Binding**—We next examined whether a cell-specific protein(s) interacts with the 60-bp intron sequence. SOX9 has been implicated in chondrogenesis because mutations in the SOX9 gene have been identified in patients with campomelic dysplasia (20, 31). SOX9 also binds to the enhancer of the first intron of the Col11a2 gene (32, 33) and the enhancer of the promoter of Col11a2 (24). Because there is one potential site for Sox9 binding (CT-CAAAAG, +1334 to +1339) within the 60-bp segment, we examined whether Sox9 interacts with this segment by incubating a 32P-labeled 60-bp probe with in vitro translated Sox9 protein in EMSA (Fig. 4A). The 60-bp probe interacted with Sox9 in a similar way to that of a positive control, 32P-labeled HMG probe that contains a consensus Sox9-binding site (AACAAAG) and has been used for protein binding to the SOX family (Fig. 3B). The complexes were competed with excess unlabeled Sox9 and 60-bp probes (Fig. 4A, lanes 3 and 4). Anti-Sox9 antibodies supershifted the 60-bp Sox9 complex (Fig. 4A, lane 5). We also examined protein-DNA interactions using nuclear extracts prepared from RCS cells. The 32P-labeled 60-bp probe formed two complexes with RCS nuclear extracts. The 32P-labeled 60-bp probe formed two complexes with RCS nuclear extracts. The 32P-labeled 60-bp probe formed two complexes with RCS nuclear extracts. The 32P-labeled 60-bp probe formed two complexes with RCS nuclear extracts.

**Identification of a Sequence for Sox9 Protein Binding**—We further delineated a sequence within the 60-bp fragment in-
involved in Sox9 binding. Six mutated double-stranded oligonucleotides, substituted with 7 bp within the 60-bp sequence, were prepared and used as competitors for complex formation of 32P-labeled 60 bp and Sox9 protein (Fig. 5A). Excess unlabeled oligonucleotides m-1, m-2, m-4, m-5, and m-6 competed with Sox9 and with 100-fold excess of unlabeled 60-bp oligonucleotides; lane 4, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and with 100-fold excess of unlabeled HMG; lane 5, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and anti-Sox9 antibodies; lane 6, 32P-labeled HMG was incubated with nuclear extracts from RCS; lane 7, 32P-labeled 60 bp oligonucleotide was incubated with nuclear extracts from RCS; lane 8, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and anti-Sox9 antibodies. B, Western blot analysis with the anti-Sox9 antibodies. lane 1, extracts from Balb 3T3 cells; lane 2, extracts from undifferentiated ATDC5 cells; lane 3, extracts from RCS cells.

**Fig. 4.** The interaction of Sox9 protein with the Col11a2 enhancer sequence by EMSA and specificity of the anti-Sox9 antibodies in Western blot. A, gel shift analyses using HMG and 60-bp oligonucleotides and in vitro translated Sox9 were performed as described under "Materials and Methods." Lanes 1 and 3, 32P-labeled HMG was incubated with Sox9; lanes 2 and 4, 32P-labeled 60-bp oligonucleotide was incubated with Sox9; lane 3, 32P-labeled HMG was incubated with Sox9 and with 100-fold excess of unlabeled 60-bp oligonucleotides; lane 4, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and with 100-fold excess of unlabeled HMG; lane 5, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and anti-Sox9 antibodies; lane 6, 32P-labeled HMG was incubated with nuclear extracts from RCS; lane 7, 32P-labeled 60 bp oligonucleotide was incubated with nuclear extracts from RCS; lane 8, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 and anti-Sox9 antibodies. B, Western blot analysis with the anti-Sox9 antibodies. Lane 1, extracts from Balb 3T3 cells; lane 2, extracts from undifferentiated ATDC5 cells; lane 3, extracts from RCS cells.

**Fig. 5.** Delineation of a CTCAAAG sequence in the 60-bp segment crucial for Sox9 binding. The interaction of 32P-labeled 60-bp oligonucleotides with Sox9 was analyzed in the presence of excess unlabeled competitors to delineate an enhancer sequence. A, the 60-bp wild type oligonucleotide sequence was shown. Mutant oligonucleotides m1–m6 contain six different 7-bp substitutions in the 60-bp sequence. Only substituted sequences are shown in the mutated oligonucleotides. B, 32P-labeled 60 bp oligonucleotide was incubated with Sox9 in the presence of various competitors. m3 failed to compete with the wild type 60-bp oligonucleotide, suggesting that sequences used for the creation of the mutation are crucial for the interaction with Sox9.

These results suggest that the 7-bp sequence is critical for enhancer activity, which is in agreement with the Sox9 binding results.

The 60-bp Fragment of Col11a2 Intron 1 Directs Cartilage-Specific Expression in Transgenic Mice—Using transgenic mice, it was previously shown that the first intron segment enhances the cartilage-specific promoter activity of Col11a2 (15). We generated transgenic mice carrying the construct 453LacZ/8 × 60 consisting of the −453 promoter and 8 × 60 bp intron of Col11a2 to test tissue-specific activity of the 60 bp intron (Fig. 6). The construct directed the expression of the reporter gene for β-galactosidase in the cartilage of embryonic 14.5-day-old mouse embryos (Fig. 6A). The scapula, humerus, ulna, and radius of the forelimbs were positive, and the primordial cartilage of the hindlimbs was also stained (Fig. 6B). A 7-bp substitution mutation in the 60-bp segment (453LacZ/8 × 60) showed no β-galactosidase expression in any tissues (Fig. 6C). These results indicate that the 60-bp intron sequence confers specifically for the transcription of Col11a2 in the cartilage and that the sequence used for the creation of the mutation contains a core sequence responsible for this tissue-specific activity.
Cartilage-specific Enhancer of Col11a2

Type XI collagen is an essential structural component in cartilage. Regulation of Col11a2 is mediated by positive and negative regulatory elements. For example, Sox9 binding elements at −600 and −520 promote cartilage-specific expression of Col11a2 (16). A neural tissue-specific element (−454 to −500) and a cartilage-specific element (−501 to −530) that converts neuronal tissue-specific expression to cartilage have been identified (13). In addition to these promoter elements, the first intron segment enhanced the promoter activity in cartilage and was required for expression of Col11a2 in the notochord (13). In this report, we have identified a sequence in the first intron required for cartilage-specific expression of Col11a2.

We found that a 300-bp intron segment contained full enhancer activity similar to that of the 2.3-kb intron segment in transfection assays in RCS cells. Transfection analysis using oligonucleotides as competitors identified a 30-bp sequence within the 300-bp segment important for the enhancer activity. This oligonucleotide competition approach has advantages over a conventional method using mutated constructs. It is quick (i.e. no requirement for the creation of mutations in the constructs) and can compare enhancer activity using the same wild type reporter construct without sacrificing the size of the enhancer segment of interest. A disadvantage may be a limitation of the size of oligonucleotides to be used as competitors.

Using this approach, we narrowed down an enhancer-containing sequence to 60 bp within the 300-bp segment. The 60-bp sequence enhanced the −453 promoter of Col11a2 by 10-fold compared with 40-fold enhancement by the 300-bp or 2-kb intron segment. The reduced enhancer activity of the 60-bp sequence may occur because its flanking sequence might be important for stable factor-DNA binding and/or recognized by other factors for the full enhancer activity in a cooperative manner. Consistent with this hypothesis, amplifications of the 60-bp sequence increased enhancer activity (i.e. 2.7, 4.6, 11.5, and 16.0-fold increase by two, four, six, and eight copies, respectively; Table I). These results suggest that multiple factors may be required for the full enhancer activity.

Gel shift assays showed that Sox9 protein bound a 7-bp sequence, CTCAAG (+1334 to +1339), within the 60-bp segment. A substitution mutation in the 7-bp sequence showed little activity in RCS cells (Table I). These results suggest that the 7-bp sequence is critical for enhancer activity through Sox9 binding. When nuclear extracts from RCS cells were used in EMSAs with the 60-bp probe, two complexes were formed. The fast migrating band represents a Sox9-DNA complex because its migration position was similar to that of the complex with the Sox9 protein and the antibodies to Sox9 supershifted the complex. It is likely that the slower migrating band contains another protein factor(s) in addition to Sox9. It was reported that other Sox family proteins (e.g. Sox5 and Sox6) cooperatively work with Sox9 for activation of the enhancer of the Col2a1 gene (34). Our observations are in agreement with these observations. These results indicate that the 7-bp cis-acting element plays a central role in the chondrocyte-specific enhancer activity and strongly suggest that the Sox9 protein is a key mediator for the transcription of Col11a2 in chondrocyte.

The 60-bp sequence was able to direct expression of the −453 promoter of Col11a2 in the cartilage of transgenic mice. The expression patterns of the reporter β-galactosidase gene were similar to those with the construct containing either the 300-bp or the 2.3-kb intron segment (15). A 7-bp substitution mutation in the 60-bp sequence eliminated the enhancer activity, suggesting that this 7-bp sequence is critical for tissue-specific activity, which is in agreement with the transfection analysis. Interestingly, the 60-bp enhancer-containing construct failed to direct β-galactosidase expression in the notochord (data not shown). Because the 2.3-kb intron segment is necessary for notochord-specific expression of Col11a2, the 60-bp sequence likely lacks the information necessary for expression in the notochord. Thus, Col11a2 is positively regulated by at least two unique activity in different tissues, and such redundancy may be necessary for the optimum expression of Col11a2 to form collagen fibrils specific to each type of cartilage. Differences in the activity of these sites observed in transfected RCS cells may not be the only regulatory mechanisms of Col11a2 expression in cartilage. Recently, it was reported that sequences adjacent to the Sox9 site are also required for the chondrocyte-specific enhancer activity of Col2a1 and that new members of the Sox family, i-Sox5 and Sox6, form a heterodimer and activate the Col2a1 enhancer co-operatively with Sox9 (34). It is conceivable that protein factors, such as i-Sox5 and Sox6, may also be involved in Col11a2 expression. Levels of Sox9 and these proteins may differ in each cartilage and have preferential utili-

DISCUSSION

Y. Liu, H. Li, K. Tanaka, N. Tsumaki, and Y. Yamada, unpublished data.
zation of the multi-modular elements containing the Sox9 site for Col11a2 expression.

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Aromatic residues in the C-terminal domain 2 are required for Nanog to mediate LIF-independent self-renewal of mouse embryonic stem cells.

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Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the α2(XI) collagen gene.

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Questions have arisen concerning the validity of Fig. 4 in this article (on page 12716). Therefore, Fig. 4 has been withdrawn by the authors.
In Fig. 6 the immunoblots against TAP1/2 (α-TAP1/2) of the single-cysteine TAP construct S179C were mistakenly transposed and have to be exchanged. However, the data presented remain factually correct. The corrected Fig. 6 is shown below.

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PAGE 12716, FIG. 4B:

Lanes 1 and 2 in the originally published Fig. 4B were duplicates of the same image. A corrected panel B is shown below.