

SOX9-dependent and -independent Transcriptional Regulation of Human Cartilage Link Protein*

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Cartilage link protein is a key component of the cartilage extracellular matrix. The transcriptional regulation of the gene encoding cartilage link protein (*CRTL1*) is largely unknown, however. Here, we investigated the regulation of *CRTL1* by SOX9, a key regulator of cartilage matrix genes and chondrogenesis. Knockdown of SOX9 resulted in decreased *CRTL1* expression. SOX9 induced *CRTL1* expression effectively in human non-chondrocytic immortalized cell lines as well as in mesenchymal stem cell and adult dermal fibroblast. These results indicate that, like other cartilage matrix genes, SOX9 is a key regulator of *CRTL1*. Unlike other cartilage matrix genes, however, the activation of *CRTL1* by SOX9 and its known transcriptional co-activators L-SOX5 and SOX6 was cell type-dependent. Two cis-acting enhancer elements resided in the 5'-untranslated region of *CRTL1*. One contained a heptameric SOX binding sequence and showed SOX9-dependent enhancer activity in several cell lines. The other showed cell type-specific SOX9-independent enhancer activity. These findings suggest that the enhancer elements may mediate differential expression of *CRTL1* during chondrocyte differentiation and maturation.

Cartilage link protein (CRT-LP)¹ is a glycoprotein that exists as a monomer of 339 amino acids (1). A major component of cartilage extracellular matrix, CRT-LP stabilizes aggregates of aggrecan and hyaluronic acid (HA). The three functional domains of CRT-LP include an Ig-fold, which interacts with the aggrecan G1 domain, and two proteoglycan tandem repeats that mediate binding to HA. CRT-LP binds aggrecan along the HA chain in a 1:1 stoichiometry (2–4). The resulting aggregates are entrapped within the mesh-like network of type II collagen fibrils, producing a large, stable macromolecular structure that contributes to compression resistance and shock absorption in the joint (2–4).

CRT-LP is expressed in numerous non-cartilaginous tissues,

such as mesonephros, brain, and sclera. However, the phenotype associated with loss of function of the CRT-LP gene, *CRTL1*, is restricted to the skeleton; *CRTL1*-null mice exhibit a perinatally lethal chondrodysplasia. The cartilage in these mice contains significantly reduced aggrecan depositions in the hypertrophic zone and decreased numbers of prehypertrophic and hypertrophic chondrocytes (5). Cartilage-specific transgene expression of *CRTL1* can completely prevent perinatal mortality in *CRTL1*-null mice and rescue skeletal abnormalities at levels dependent upon the amount of CRT-LP expression (6). In addition, CRT-LP may function as a growth factor to up-regulate the synthesis of aggrecan and type II collagen in cartilage (7). Thus, production of CRT-LP at appropriate levels is crucial to the formation of proteoglycan aggregates and the normal organization of hypertrophic chondrocytes. To date, however, the mechanisms that control *CRTL1* expression remain largely unknown.

A key transcriptional regulator of chondrogenesis is SOX9, a member of the SOX (Sry-type HMG box) family, that is characterized by a reduced amino acid sequence within the HMG (high mobility group) domain (8, 9). Prechondrogenic mesenchyme cells with a homozygous deletion of *SOX9* are unable to differentiate into chondrocytes and cannot express chondrocyte-specific matrix genes such as *Col2a1*, *Col9a2*, *Col11a2*, and *Agc1* (10). In developing mouse embryos, *SOX9* expression closely parallels that of *Col2a1* (11, 12). SOX family proteins bind to DNA through a heptameric enhancer motif, (A/T)(A/T)CAA(A/T)G (13, 14). SOX9 has been demonstrated to regulate expression of *Col2a1* via several of these heptameric motifs (15). Other cartilage matrix genes, including *Col9a1*, *Col9a2*, *Col11a2*, *CD-Rap*, and *Agc1*, have also been identified as direct targets of SOX9 (16–20). These findings indicate that cartilage genes might have common mechanisms for transcriptional regulation and suggest a role for SOX9 in regulating *CRTL1* expression.

In this study, we have shown that the 5'-untranslated region (5'-UTR) of *CRTL1* contains a cis-acting element that is directly regulated by SOX9. SOX9 can induce *CRTL1* expression in human non-chondrocytic cell lines, mesenchymal stem cells, and adult dermal fibroblasts. We also show that the 5'-UTR of *CRTL1* contains another cis-acting element that responds in a cell type-specific manner and is independent of SOX9.

MATERIALS AND METHODS

Cell Culture—HuH-7 cells were obtained from the Riken Cell Bank (Tsukuba, Japan), and HeLa cells were obtained from the Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan). HEK293 cells were purchased from Clontech (Palo Alto, CA). All cell lines were cultured at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM)-high glucose supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 10% fetal bovine serum. OUMS-27 cells were obtained from the Institute for Fermentation, Osaka Animal Cell Bank (Osaka, Japan) and cultured in DMEM-high glucose supplemented with kanamycin (50 µg/ml) and 10% fetal bovine serum. Hu-

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¹ The abbreviations used are: CRT-LP, cartilage link protein; UTR, untranslated region; DMEM, Dulbecco's modified Eagle's medium; hMSC, human mesenchymal stem cell; hDFB, human adult dermal fibroblast; RNAi, RNA interference; EMSA, electrophoretic mobility shift assay; WT, wild type; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

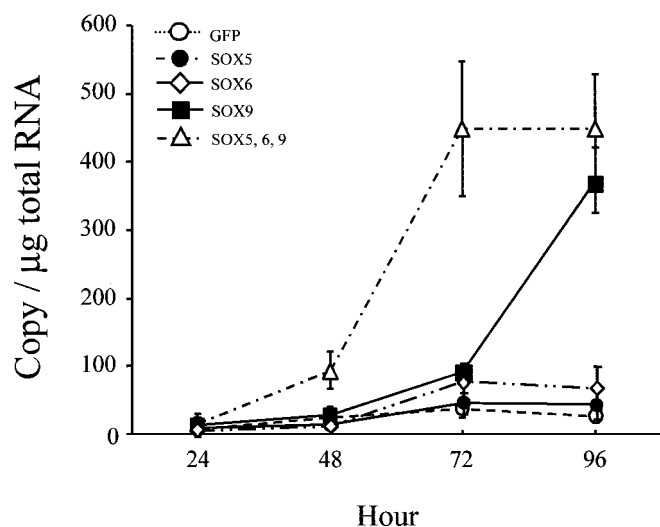


FIG. 1. *CRTL1* is induced by SOX9 in the non-chondrocytic cell line HuH-7. Total RNA was isolated from HuH-7 cells following transient transfection of green fluorescent protein (open circles), SOX5 (closed circles), SOX6 (open diamonds), SOX9 (closed squares), and SOX9 with SOX5 and SOX6 (open triangles). The endogenous *CRTL1* mRNA levels were quantified by SYBR Green real-time PCR and normalized to *GAPDH* mRNA. The values represent the means \pm S.D. of quadruplicate measurements.

man mesenchymal stem cells (hMSC) and human adult dermal fibroblasts (hDFB) were purchased from Cambrex (East Rutherford, NJ). The hMSC line was cultured in MSC growing medium, and the hDFB line was cultured in DMEM-high glucose supplemented with penicillin (50 units/ml), streptomycin (50 μ g/ml), and 10% fetal bovine serum. For all cultures, the growth medium was replaced every 3–4 days.

SOX Plasmids and Adenoviruses—Full-length cDNAs of SOX9, L-SOX5, and SOX6 were amplified by PCR. DNA sequences of PCR products were verified by automated DNA sequencing (model 3700; ABI, Foster City, CA) and then cloned into pEGFP-C1 or pShuttle mammalian expression vectors (Clontech). Adenoviral expression vectors containing L-SOX5, SOX6, and SOX9 were constructed using the AdenoX expression system (Clontech) according to the manufacturer's instructions. The adenoviruses were packed and amplified in HEK293 cells and then purified using the AdenoX virus purification kit (Clontech). Virus titers were estimated using the AdenoX rapid titer assay kit (Clontech).

Induction of Adenoviruses into hMSC and hDFB—Confluent hMSC and hDFB cultures were transfected with SOX-expressing adenoviruses at a multiplicity of infection of 50. SOX-induced hMSC and hDFB cells were collected 2 days after transduction and gently centrifuged into pellets (5×10^5 cells/pellet). Cell pellets were cultured in serum-free high-glucose DMEM. After 3, 7, 14, and 21 days of pellet culture, mRNA was harvested from cell pellets, and gene expression levels were measured using a real-time PCR assay. Three-dimensional cultures on collagen gel matrices were produced using the 3D-Collagen Cell Culture System (Chemicon, Temecula, CA). Two days after transduction, SOX-transduced hMSC and hDFB cells were seeded onto DMEM-containing collagen gel matrices at a density of 2.5×10^5 cells/cm² in 24-well tissue culture plates. After 7, 14, and 21 days of three-dimensional culture in serum-free high-glucose DMEM, mRNA was harvested from cells and subjected to real-time PCR.

RNA Interference (RNAi)—The pSilencer2.1-U6 neo expression vector kit (Ambion, Austin, TX) was used according to the manufacturer's protocol. RNAi target sequences for human *SOX9* mRNA were selected using a small interfering RNA target design online tool (www.takarabio.co.jp/rnai/intro.htm) and were analyzed by a BLAST search to ensure the gene specificity. Several small interfering RNA constructs were tested, and the most effective one was used. The target sequence was 5'-AACTCCAGCTCCTACTACAGC-3'. OUMS-27 cells were plated at a density of 5×10^4 cells/well in 12-well tissue culture plates. Using FuGENE 6 (Roche Applied Science), 1 μ g of a small interfering RNA construct or an empty vector was transfected. Two days after transfection, mRNAs were harvested from two independent wells and subjected to real-time PCR.

Real-time Quantitative PCR Assays—Total RNAs from cells were isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and

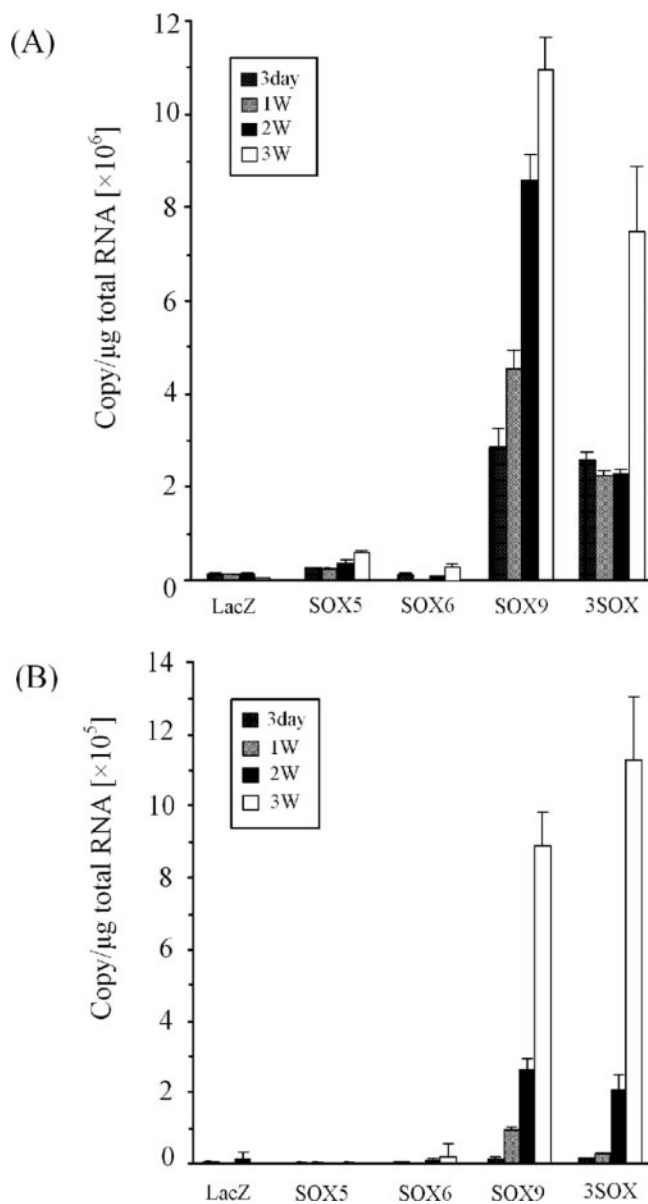


FIG. 2. *CRTL1* is induced by SOX genes in hMSC and hDFB in pellet culture. Transduction of adenoviruses with L-SOX5, SOX6, and SOX9, each or in combination (3SOX), in hMSC (A) and hDFB (B) cells. *CRTL1* mRNA levels were quantified by SYBR Green real-time PCR and normalized to *GAPDH* mRNA. The values represent the means \pm S.D. of quadruplicate measurements.

treated with DNaseI. Total RNAs (50 ng-1 μ g) were reverse transcribed into cDNA using the Taqman core reagent kit (ABI) according to the manufacturer's protocol. Subsequently, 1 μ l of each room temperature reaction was used as a template for the second step of SYBR Green reverse transcribed PCR (Qiagen). A partial cDNA of *CRTL1* and *SOX9* was amplified by PCR (using the primer sequences 5'-TCACACAGAG-GTGGCAATGT-3' and 5'-TGCCCTCCCTTCAGAAACACT-3', 5'-CAT-GAGCGAGGTGCACTCC-3' and 5'-TCGCTTCAGGTCAGCCTTG-3', respectively) and cloned into the pCR-TOPOII vector (Invitrogen). SYBR Green PCR amplification and real-time fluorescence detection were performed using the ABI PRISM 7700 sequence detection system. PCR cycling conditions were as follows, 94 $^{\circ}$ C for 15 min followed by 40 cycles at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s.

Construction of *CRTL1* Plasmids—*CRTL1*-luciferase fusion genes were constructed in the pGL3-Basic vector (Promega, Madison, WI). All *CRTL1* fragments were amplified by PCR using a primer pair that contained MluI- or XhoI-cleaved restriction sites at the 5'-ends. The 35-bp wild type and mutant fragments were synthesized as double-stranded oligonucleotides containing MluI-cleaved sites at both ends (Sigma Genosis, Tokyo, Japan). These fragments were multimerized to

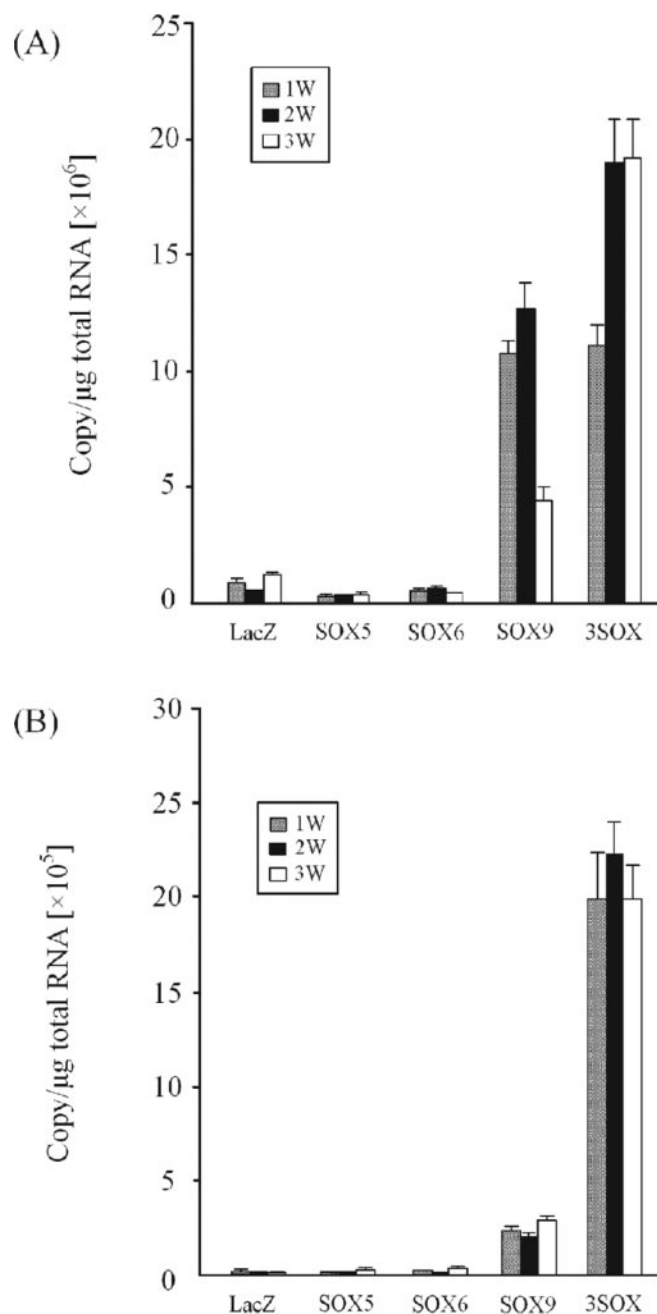


FIG. 3. *CRTL1* is induced by SOX genes in hMSC and hDFB in three-dimensional culture. Transduction of adenoviruses with L-SOX5, SOX6, and SOX9, each or in combination (3SOX), in hMSC (A) and hDFB (B) cells. *CRTL1* mRNA levels were quantified by SYBR Green real-time PCR and normalized to *GAPDH* mRNA. The values represent the means \pm S.D. of quadruplicate measurements.

create sequences of 2–4 tandem repeats (Fig. 6). All plasmid constructs were verified by DNA sequencing.

Luciferase Reporter Assays—Plasmid DNA transfections were performed using FuGENE 6 (Roche Applied Science). Cells were plated at a density of 5×10^4 cells/well in 24-well tissue culture plates. Luciferase reporter plasmids were co-transfected with the pRL-TK plasmid as an internal control for transfection efficiency. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured using the PG-DUAL-SP reporter assay system (TOYO Ink, Tokyo, Japan). Relative transcriptional activity was expressed as a ratio of luciferase reporter gene activity from the experimental vector to that from the internal control vector.

Electrophoretic Mobility Shift Assays (EMSAs)—Wild type (WT) and mutant (m-1) probes were prepared by annealing 35-bp complementary oligonucleotides labeled with digoxigenin (DIG)-11-ddUTP. DNA-protein binding reactions were performed using a DIG gel shift

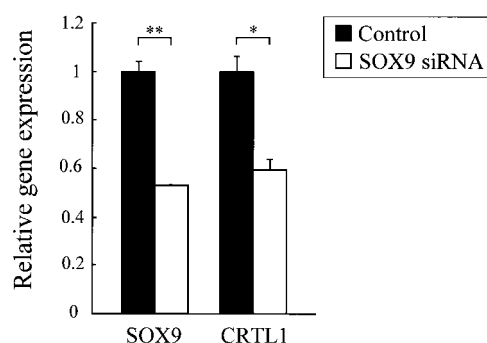


FIG. 4. RNAi for *SOX9* reduced *CRTL1* transcription. OUMS-27 cells were transfected with a pSilencer expression vector containing a 21-nucleotide hairpin RNA corresponding to *SOX9* mRNA or with a control vector. RNAs were prepared from transfected cells after 2 days. *SOX9* and *CRTL1* mRNAs were quantified by SYBR Green real-time PCR and normalized to *GAPDH* mRNA. The values represent the means \pm S.D. of quadruplicate measurements and are shown as a ratio of expression level obtained with the control vector. **, $p < 0.01$; *, $p < 0.05$ by unpaired Student's *t* test.

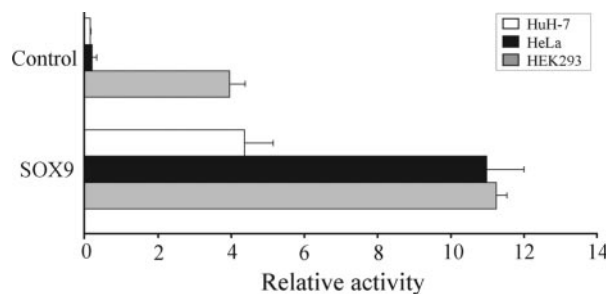


FIG. 5. The 1.2-kb fragment containing the promoter and 5'-untranslated regions of *CRTL1* is transcriptionally activated by SOX9 in non-chondrocytic cell lines. The reporter plasmid pCRTL1 was co-transfected with SOX9 into HuH-7 (white), HeLa (black), and HEK293 (gray) cells.

kit (Roche Applied Science). SOX9 protein was synthesized using the TNT transcription/translation system (Promega), and nuclear extracts from various cell lines were prepared as previously described (21). Supershift experiments were performed with purified SOX9 antibody (provided by Drs. Tomoatsu Kimura, Toyama Medical and Pharmaceutical University, and Yoshihiko Yamada, NIDCR, National Institutes of Health) (22). Competition experiments were performed by preincubating *in vitro* translated SOX9 or nuclear extract with excess unlabeled oligonucleotides prior to adding labeled oligonucleotides. Reaction mixtures were incubated for 20 min at room temperature. DNA-protein complexes were resolved on 6% Tris-boric acid-EDTA gels (Invitrogen), and signal was detected using a chemiluminescent detection system (Roche Applied Science).

RESULTS

***CRTL1* Induction by SOX9 in Human Non-chondrocytic Immortalized Cell Lines**—We examined the effect of SOX9 on *CRTL1* expression in the human non-chondrocytic cell line HuH-7. Endogenous *CRTL1* expression is undetectable in this cell line.² Transient transfection of plasmids expressing green fluorescent protein-tagged SOX genes into HuH-7 cells produced easily detectable levels of SOX protein expression, with peak expression observed 48–72 h following transfection (data not shown). In SOX9-transfected cells, endogenous *CRTL1* expression increased gradually beginning at 48 h after transfection and was remarkably elevated from 72 until 96 h after transfection (Fig. 1).

Previous studies of other cartilage matrix genes, including *Col2a1*, *Col11a2*, and *Agc1*, have shown that the transcriptional activity of SOX9 is enhanced by the co-activators L-

² I. Kou and S. Ikegawa, unpublished data.

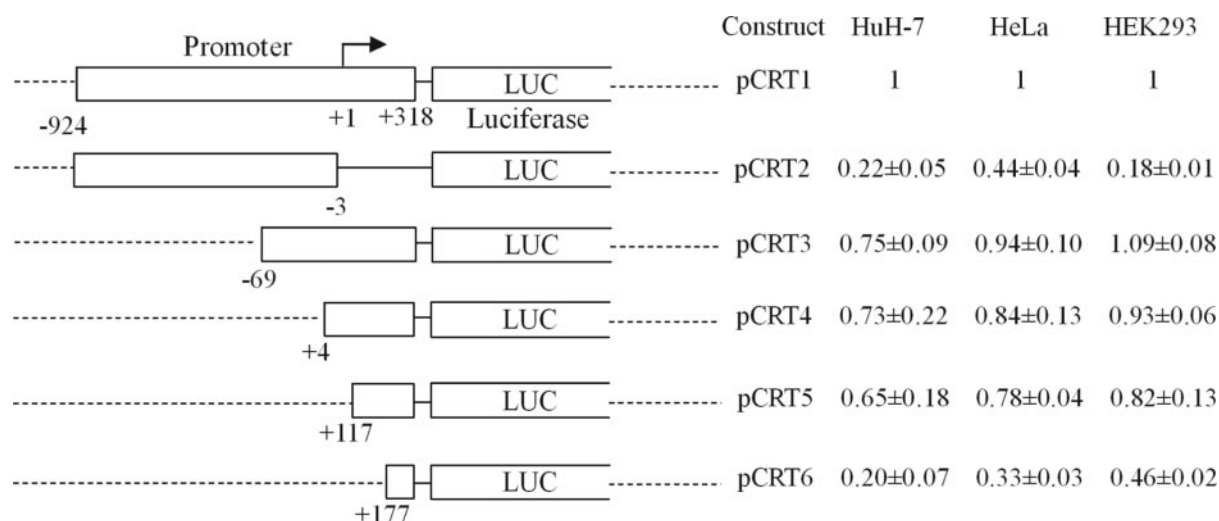


FIG. 6. Localization of the *CRTL1* promoter/enhancer activity within the 1.2-kb fragment. *CRTL1* reporter gene constructs and their luciferase activities in the presence of transiently transfected SOX9. Various sizes of the promoter/enhancer fragments (white boxes) were cloned upstream of the luciferase gene in the pGL3 reporter vector. The positions of their first and last nucleotides relative to the transcription start site (+1) of the *CRTL1* gene (NM_001884.1) are indicated below the boxes. Luciferase activities are presented as means \pm S.D. and are normalized to 1 for the activity obtained with the pCRT1 construct. Results are shown for four independent cultures tested in two representative experiments.

SOX5 and SOX6 (23–25). Therefore, we investigated whether L-SOX5 and SOX6 could enhance the effect of SOX9 on *CRTL1* expression. In cells co-expressing SOX9, L-SOX5, and SOX6, *CRTL1* expression increased sooner, beginning at 24 h after transfection (Fig. 1). However, both L-SOX5 and SOX6 appear to have no direct effect on *CRTL1* expression. These results suggest that SOX9 can regulate endogenous *CRTL1* in addition to other major cartilage matrix genes and that L-SOX5 and SOX6 may enhance the transcriptional activity of SOX9.

***CRTL1* Induction by SOX9 in hMSC and hDFB Cells**—To confirm SOX9-mediated regulation of *CRTL1* expression in other cell lines, we evaluated the effect of SOX9 on *CRTL1* expression in hMSC cells. Two days after adenovirus transduction of SOX9, hMSCs were collected into small pellets and cultured in serum-free growth medium. We detected an increase in *CRTL1* mRNA expression 3 days after SOX9 transduction, and expression levels remained elevated for 3 weeks following transduction. We observed similar patterns of *CRTL1* mRNA expression in cells co-transduced with SOX9, L-SOX5, and SOX6 (Fig. 2A).

In addition, we examined whether *CRTL1* could be induced by SOX9 in well differentiated mesenchymal cells such as hDFB. In hDFB cells, *CRTL1* expression was first induced at Day 3 of pellet culture, and expression levels remained increased for 3 weeks (Fig. 2B). We further examined *CRTL1* induction in hMSC and hDFB cells using a three-dimensional culture system. Interestingly, SOX9 with L-SOX5 and SOX6 could co-activate *CRTL1* expression more strongly than SOX9 alone (Fig. 3). These results indicate that SOX9 is a key regulator of *CRTL1* and that *CRTL1* can be induced by SOX9 in both differentiated and undifferentiated human mesenchymal cells.

Reduction of *CRTL1* Expression by RNAi for SOX9—To test whether SOX9 is necessary for *CRTL1* expression, we inhibited SOX9 expression using small interfering RNA in a human chondrosarcoma cell line, OUMS-27, which normally expresses high levels of SOX9 and *CRTL1* (data not shown). OUMS-27 cells were transfected with an RNAi expression plasmid or a control vector. *GAPDH* mRNA levels were similar in test cells and mock controls (data not shown). The SOX9 RNAi reduced SOX9 mRNA levels to \sim 50% of the original level and resulted in inhibition of *CRTL1* expression (Fig. 4). Thus, SOX9 is necessary to maintain expression of *CRTL1*.

Transcriptional Activity of the 5'-Flanking Sequence of *CRTL1* in Non-chondrocytic Cells—To examine whether *CRTL1* transcription is directly regulated by SOX9, we measured the effect of SOX9 expression on transcriptional activity from the 5'-flanking sequence of the *CRTL1* gene. We performed transient transfection assays in several non-chondrocytic cell lines (HuH-7, HeLa, and HEK293) using the construct pCRT1, which contains \sim 1.2 kb of the 5'-flanking sequence (–924 to +318) of the *CRTL1* gene (NM_001884.1). In cells co-transfected with the SOX9 plasmid, transcriptional activity from the pCRT1 plasmid was greatly increased relative to control experiments (Fig. 5). This observation suggests that the transcriptional activity of *CRTL1* is directly regulated by SOX9 and that a regulatory region resides within the 1.2-kb region. Next, we tested the effect of SOX9 on pCRT2, which contains a 3'-deletion of the flanking sequence (–924 to –3). Transcriptional activity from pCRT2 was considerably lower than that from pCRT1 in cells co-transfected with SOX9, further indicating that a SOX9-responsive element resides within the 3'-segment of the 1.2-kb enhancer fragment (–2 to +318), the 5'-UTR of the *CRTL1* gene (Fig. 6).

To localize the cis-element within the 5'-UTR, we constructed plasmids containing various 5'-deletions of the \sim 400-bp enhancer fragment (–69 to +318). A 5'-deletion to +117 retained almost complete enhancer activities, whereas a 5'-deletion to +177 showed remarkably decreased activity (Fig. 6). These experiments localized the minimal enhancer element to the sequence between nucleotides +117 and +177. Sequence analysis of this region identified a motif that is identical to consensus SOX binding sequences, with the exception of a 1-bp mismatch (Fig. 7A, CACAAAG, +120 to +126). Next, we tested the SOX9-dependent enhancer activity of multiple, tandemly arranged copies of the 35-bp enhancer element containing the putative SOX binding motif. In all cell lines co-transfected with the SOX9 plasmid, SOX9-dependent activity increased in correlation with the number of enhancer element repeats (Fig. 7B). Substitution of a 3-bp sequence within the motif abolished enhancer activity, indicating that this sequence is critical to SOX activity (Fig. 7, A and C).

Binding of SOX9 to the *CRTL1* Enhancer Sequence—We used EMSA to determine whether SOX9 binds the 35-bp 5'-UTR cis-element directly. Incubation of a DIG-labeled WT probe with *in vitro* translated SOX9 protein resulted in the

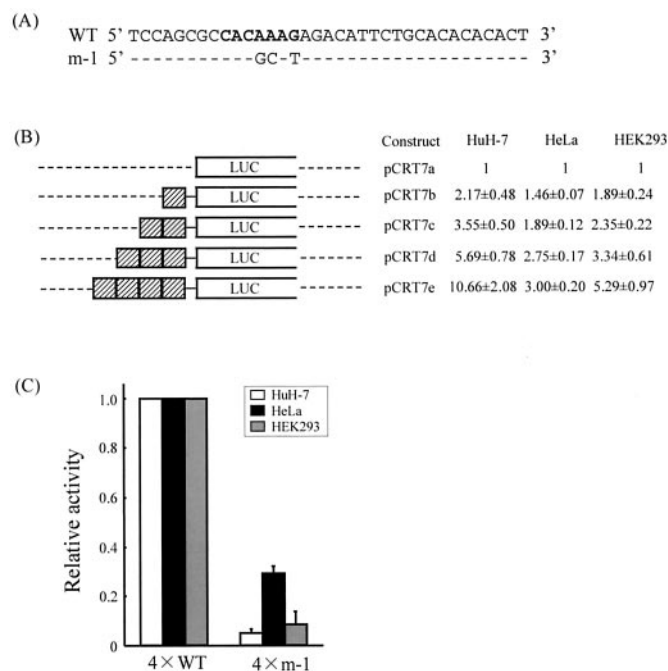


FIG. 7. Transcriptional activity of the 35-bp enhancer fragment depends on the consensus SOX-binding site. A, nucleotide sequences of the 35-bp enhancer element (WT) and the mutated element (m-1). Only mutated nucleotides are indicated; unchanged nucleotides are represented by dashes. The putative SOX-binding site is in bold. B, enhancer activities are increased by multimerization of the 35-bp element (shaded box) in three non-chondrocytic cell lines. Luciferase activities are presented as means \pm S.D. and are normalized to 1 for the activity obtained with the pGL3 vector. Results are shown for four independent cultures tested in two representative experiments. C, mutation in the SOX-binding site abolishes transcriptional activity of the 35-bp enhancer fragments. Four copies of the wild type (WT) and mutated (m-1) fragments were cloned upstream of the luciferase reporter gene. Transcriptional activities of both fragments were examined by transient transfection in three non-chondrocytic cell lines, HuH-7 (white), HeLa (black), and HEK293 (gray). Luciferase activities are presented as means \pm S.D. and are normalized to 1 for the activity obtained with four repeats of the wild type 35-bp element. Results are shown for four independent cultures tested in two representative experiments.

formation of a DNA-protein complex that could be competed with an excess amount of unlabeled probe (Fig. 8A). Mutated probes containing the 3-bp substitution within the enhancer element were unable to form complexes (Fig. 8B). Incubation with a SOX9-specific monoclonal antibody produced a super-shift of the DNA-SOX9 complex (Fig. 8A, lane 2). Together, these observations indicate that the 35-bp fragment interacts specifically with SOX9 through the consensus SOX9 binding motif.

Function of the SOX9-dependent Enhancer Element in a Chondrocytic Cell Line—To examine whether the SOX9-dependent enhancer element is functional in chondrocytes, we performed transient transfection assays using a human chondrosarcoma cell line, OUMS-27. In the experiment, the 35-bp SOX9-dependent element showed enhanced transcriptional activity, which was abolished by mutation of the SOX9 binding motif (Fig. 9). This result suggests that the SOX9-dependent enhancer element is involved in the regulation of *CRTL1* expression in chondrocytes.

Identification and Characterization of the SOX9-independent Enhancer Element—During the course of our examination of regulatory elements within the 5'-UTR, we tested the effects of sequential 3'-deletions within this sequence. Deletion of ~100-bp from the 3'-end significantly decreased enhancer activity (data not shown). This deleted region contains four pu-

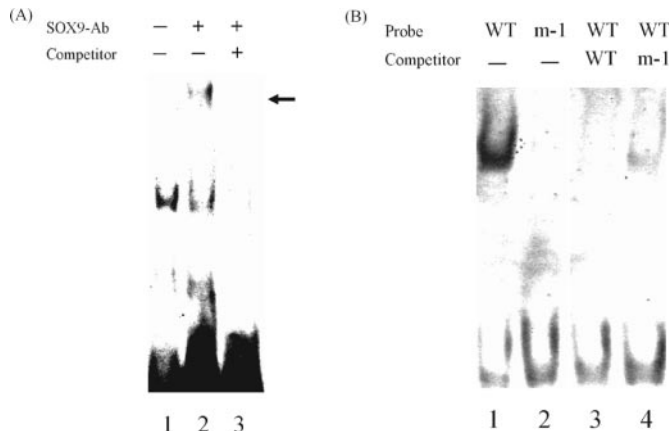


FIG. 8. Binding of SOX9 protein to the *CRTL1* enhancer sequence. A, EMSA using the 35-bp enhancer oligonucleotide (probe) and *in vitro* translated SOX9. A DIG-labeled probe was incubated with SOX9 (lane 1), SOX9 and anti-SOX9 antibody (lane 2), and SOX9 and a 125-fold excess of unlabeled probe (lane 3). Arrow indicates super-shifted SOX9 enhancer complex. B, EMSA using *in vitro* translated SOX9 with the wild type (WT, lane 1) and mutated (m-1, lane 2) probes. Competition analyses were performed using 125-fold excess of the unlabeled 35-bp WT (lane 3) and m-1 (lane 4) probe as competitor.

tative SOX binding motifs. Therefore, we hypothesized that a second enhancer element might exist within this region.

To localize and characterize this potential element, we created multimers of two sequence fragments, each of which contained two of the four putative SOX binding motifs. We cloned the multimers downstream of a *CRTL1* promoter (-336 to -3) that was not enhanced by SOX9 (data not shown) and then tested their transcriptional activity in three non-chondrocytic cell lines. A 40-bp enhancer element (+218 to +257) exhibited activity that was most pronounced in HEK293 cells; this activity increased depending on copy number but was independent of SOX9 (Fig. 10, A and B). These results suggest the presence of a SOX9-independent cis-element in the 40-bp fragment that responds to some transcription factors expressed specifically in HEK293 cells. Sequence analysis of the 40-bp fragment identified no known binding motifs, aside from the SOX motifs, for transcription factors involved in regulating chondrogenesis.

To determine whether the SOX9-independent enhancer element is functional in chondrocytes, we performed transient transfection assays using OUMS-27 cells and found the element was active in the cells (Fig. 10C). This result suggests the SOX9-independent enhancer element is also involved in the regulation of *CRTL1* expression in chondrocytes.

To examine the basis for the cell type-specific activity of the SOX9-independent element, we performed EMSA assays using nuclear extracts from various cell lines. Four DNA-protein complexes were found in the nuclear extracts from HEK293 cells (Fig. 11A). Complex 3 was found in HEK293 and OUMS-27 cells where the SOX9-independent enhancer element was active, whereas it was barely visible in HuH-7 and HeLa cells where the element was inactive. The binding of the DNA-protein complexes was specific (Fig. 11B). Therefore, it is likely that some transcription factor(s) that is specifically expressed in HEK293 and OUMS-27 cells binds to the 40-bp enhancer element and transactivates *CRTL1* independent of SOX9.

DISCUSSION

We have demonstrated that SOX9 can regulate the transcriptional activity of *CRTL1* through a heptameric SOX-binding site within the 5'-UTR. Other major cartilage matrix genes, including *Col2a1*, *Col11a2*, and *Agc1*, also are regulated by SOX9 through heptameric SOX binding elements (16, 18, 26).

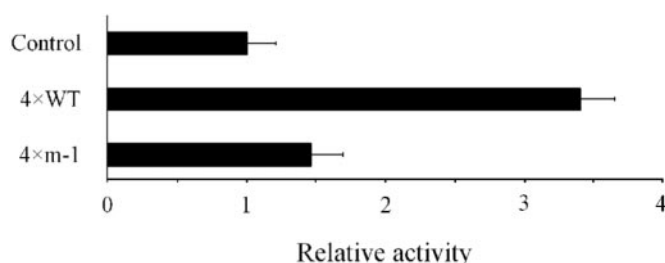


FIG. 9. **The SOX9-dependent enhancer fragments regulate *CRTL1* transcription in OUMS-27 cells.** Four tandem repeats of WT or mutant (*m-1*) enhancer elements were used for transient transfection. Luciferase activities are presented as means \pm S.D. and are normalized to 1 for the activity obtained with the pGL3 vector. Results are shown for four independent cultures tested in two representative experiments.

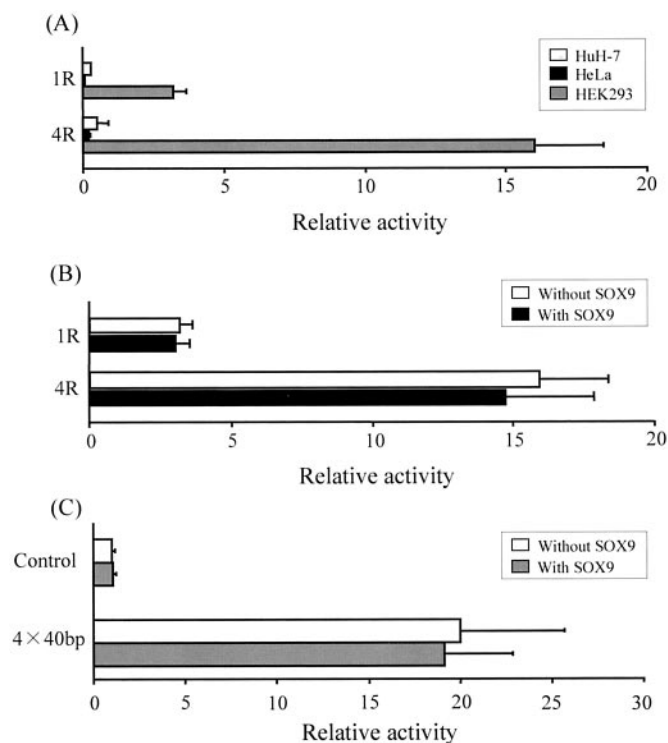


FIG. 10. **A SOX9-independent 40-bp enhancer fragment in the 5'-UTR also regulates *CRTL1* transcription.** A, the enhancer activity of the 40-bp fragment is cell type-specific. The enhancer element was multimerized one (1R) to four repeats (4R) at the downstream of the *CRTL1* promoter (-336 to -3), and the constructs were transiently transfected into three non-chondrocytic cell lines, HuH-7 (white), HeLa (black), and HEK293 (gray). B, the enhancer activity of the fragment is independent of SOX9. The multimerized 40-bp fragments were transiently transfected with or without SOX9 in HEK293 cells. C, the SOX9-independent enhancer regulates *CRTL1* transcription in OUMS-27 cells. Four tandem repeats of 40-bp fragments were transiently transfected with or without SOX9. The enhancer activity of the 40-bp fragment is also independent of SOX9 in OUMS-27 cells. Luciferase activities are presented as means \pm S.D. Results are shown for four independent cultures tested in two representative experiments.

Thus, SOX9-dependent regulation is considered to be a common mechanism for transcriptional regulation of cartilage matrix genes. Most SOX9 binding elements in the *Col2a1*, *Col11a2*, and *Agc1* genes are localized to the first intron within each gene. In contrast, the intron 1 sequence of *CRTL1* contains no similar regulatory regions and is not highly conserved between species. Comparison of the human and mouse genomic sequences revealed that the SOX9-dependent cis-element within 5'-UTR is highly conserved (more than 90% identity), suggesting that transcriptional regulation of *CRTL1* by SOX9

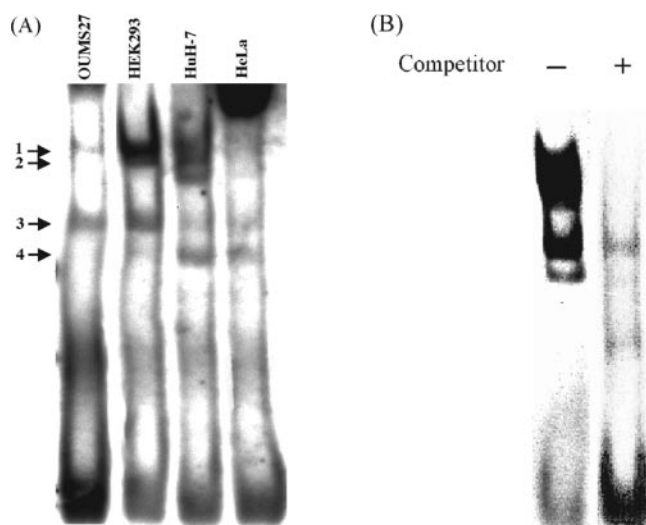


FIG. 11. **EMSA of the 40-bp SOX9-independent element.** A, EMSA using nuclear extracts from various cell lines shows the different binding patterns. Four major DNA-protein complexes identified in the extracts from HEK293 cells were numbered. Complex 3 was found in HEK293 and OUMS-27 cells, whereas it was barely visible in HuH-7 and HeLa cells. B, specificity of the DNA-protein complexes identified in the extracts from HEK293 cells. DIG-labeled probe and the cell extracts were incubated without (lane 1) or with (lane 2) 125-fold excess of the unlabeled element.

is a common mechanism between species.

SOX9 appears to function as the master regulator of *CRTL1*. Our RNAi analysis shows that SOX9 is necessary for *CRTL1* expression. We also found that SOX9 effectively induces *CRTL1* in human cells, even in non-chondrogenic cell lines and in differentiated and undifferentiated mesenchymal cells. However, we observed a delay between the time of SOX9 transduction and induction of *CRTL1* mRNA expression. This finding suggests the presence of additional factor(s) that are regulated by SOX9 and mediate SOX9 function. Likely candidates for this role include the SOX9 transcriptional co-activators L-SOX5 and SOX6, which are known to be induced by SOX9 and cooperate with SOX9 to induce *COL2A1* expression (11, 27, 28). Our present experiments using HuH-7 cells support this suggestion, as *CRTL1* induction in cells transduced with SOX9, L-SOX5, and SOX6 began earlier than that in cells expressing SOX9 alone. By Day 4, however, *CRTL1* induction levels were equivalent under both experimental conditions.

This effect of L-SOX5 and SOX6 to SOX9 is not consistent among all cartilage matrix genes, however. For example, SOX9-mediated transcription from the *COL9A1* promoter is not stimulated by L-SOX5 and SOX6 (19). In the pellet culture of hMSC and hDFB cells, we observed no additive effect of L-SOX5 and SOX6 to SOX9 activity, whereas SOX9 expression itself was sufficient for *CRTL1* induction. In contrast, L-SOX5 and SOX6 have a marked additive effect on SOX9 activity in the three-dimensional culture of hDFB and MSC cells. Our findings indicate that the regulatory mechanism of SOX9 will differ between genes and is likely specific to cell type and/or stages of differentiation.

We have also demonstrated the presence of another sequence element within the 5'-UTR that shows strong, SOX9-independent enhancer activity. In contrast to the SOX9-dependent element, the activity of the element is cell type-specific. Binding experiments using nuclear extracts from chondrocytic and non-chondrocytic cell lines showed a cell type-specific binding pattern with a DNA-protein complex that was specific to the cells where the SOX9-independent element is active. These obser-

variations suggest that the difference in enhancer activity between the cell lines results from the difference in the trans-factor(s) in the cells. In this context, original tissues of OUMS-27 and HEK293 cells are chondrocyte and kidney, respectively, where the *CRTL1* expression is strong (27). Therefore, we speculate that one or more factors in addition to SOX9 contribute to transcriptional regulation of *CRTL1*. The expression profile of *CRTL1* and *SOX9* during chondrocyte proliferation and differentiation is consistent with this idea; *CRTL1* is expressed in hypertrophic chondrocytes, where *SOX9* expression is no longer detectable (12, 28–30). In addition, the *SOX9* enhancer elements within the *Col2a1* and *Col11a2* genes have been shown to bind protein(s) other than *SOX9* (15, 16). The presence of a glucocorticoid-like response element in intron 1 of the human and rat *CRTL1* genes has been reported (31, 32) as well as a cell type-specific AT-rich element within the *CRTL1* promoter region that binds multiple transcription factors (32). Comparison of the human and mouse genomic sequences revealed that the *SOX9*-independent cis-element within 5'-UTR is also highly conserved (~80% identity), suggesting that this regulatory region has an important role in the transcriptional mechanism of *CRTL1*. Further study is necessary to clarify regulator(s) of *CRTL1* other than *SOX9*.

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REFERENCES

- Neame, P. J., Christner, J. E., and Baker, J. R. (1986) *J. Biol. Chem.* **261**, 3519–3535
- Neame, P. J., and Barry, F. P. (1993) *Experientia* **49**, 393–402
- Morgelin, M., Paulsson, M., Hardingham, T. E., Heinegard, D., and Engel, J. (1988) *Biochem. J.* **253**, 175–185
- Dudhia, J., Bayliss, M. T., and Hardingham, T. E. (1994) *Biochem. J.* **303**, Pt. 1, 329–333
- Watanabe, H., and Yamada, Y. (1999) *Nat. Genet.* **21**, 225–229
- Czipri, M., Otto, J. M., Cs-Szabo, G., Kamath, R. V., Vermes, C., Firneisz, G., Kolman, K. J., Watanabe, H., Li, Y., Roughley, P. J., Yamada, Y., Olsen, B. R., and Glant, T. T. (2003) *J. Biol. Chem.* **278**, 39214–39223
- McKenna, L. A., Liu, H., Sansom, P. A., and Dean, M. F. (1998) *Arthritis Rheum.* **41**, 157–162
- Wegner, M. (1999) *Nucleic Acids Res.* **27**, 1409–1420
- Pevny, L. H., and Lovell-Badge, R. (1997) *Curr. Opin. Genet. Dev.* **7**, 338–344
- Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrughe, B. (1999) *Nat. Genet.* **22**, 85–89
- Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P., and Cheah, K. S. (1997) *Nat. Genet.* **16**, 174–178
- Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997) *Dev. Biol.* **183**, 108–121
- Harley, V. R., Lovell-Badge, R., and Goodfellow, P. N. (1994) *Nucleic Acids Res.* **22**, 1500–1501
- Sudbeck, P., Schmitz, M. L., Baeuerle, P. A., and Scherer, G. (1996) *Nat. Genet.* **13**, 230–232
- Zhou, G., Lefebvre, V., Zhang, Z., Eberspaecher, H., and de Crombrughe, B. (1998) *J. Biol. Chem.* **273**, 14989–14997
- Bridgewater, L. C., Lefebvre, V., and de Crombrughe, B. (1998) *J. Biol. Chem.* **273**, 14998–15006
- Xie, W. F., Zhang, X., Sakano, S., Lefebvre, V., and Sandell, L. J. (1999) *J. Bone Miner. Res.* **14**, 757–763
- Sekiya, I., Tsuji, K., Koopman, P., Watanabe, H., Yamada, Y., Shinomiya, K., Nifuji, A., and Noda, M. (2000) *J. Biol. Chem.* **275**, 10738–10744
- Zhang, P., Jimenez, S. A., and Stokes, D. G. (2003) *J. Biol. Chem.* **278**, 117–123
- Bernard, P., Tang, P., Liu, S., Dewing, P., Harley, V. R., and Vilain, E. (2003) *Hum. Mol. Genet.* **12**, 1755–1765
- Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- Liu, Y., Li, H., Tanaka, K., Tsumaki, N., and Yamada, Y. (2000) *J. Biol. Chem.* **275**, 12712–12718
- Lefebvre, V., Li, P., and de Crombrughe, B. (1998) *EMBO J.* **17**, 5718–5733
- de Crombrughe, B., Lefebvre, V., Behringer, R. R., Bi, W., Murakami, S., and Huang, W. (2000) *Matrix Biol.* **19**, 389–394
- Lefebvre, V., Behringer, R. R., and de Crombrughe, B. (2001) *Osteoarthr. Cart.* **9**, Suppl. A, S69–S75
- Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N., and de Crombrughe, B. (1997) *Mol. Cell. Biol.* **17**, 2336–2346
- Binette, F., Cravens, J., Kahoussi, B., Haudenschild, D. R., and Goetinck, P. F. (1994) *J. Biol. Chem.* **269**, 19116–19122
- Vornehm, S. I., Dudhia, J., Von der Mark, K., and Aigner, T. (1996) *Matrix Biol.* **15**, 91–98
- Zhao, Q., Eberspaecher, H., Lefebvre, V., and De Crombrughe, B. (1997) *Dev. Dyn.* **209**, 377–386
- Chen, Q., Johnson, D. M., Haudenschild, D. R., and Goetinck, P. F. (1995) *Dev. Biol.* **172**, 293–306
- Rhodes, C., Savagner, P., Line, S., Sasaki, M., Chirigos, M., Doege, K., and Yamada, Y. (1991) *Nucleic Acids Res.* **19**, 1933–1939
- Rhodes, C., and Yamada, Y. (1995) *Nucleic Acids Res.* **23**, 2305–2313