THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 292, NO. 2, pp. 528–538, January 13, 2017

A Novel Regulatory Mechanism of Type II Collagen Expression via a SOX9-dependent Enhancer in Intron 6*

Received for publication, September 13, 2016, and in revised form, November 17, 2016 Published, JBC Papers in Press, November 23, 2016, DOI 10.1074/jbc.M116.758425

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Edited by Eric R. Fearon

Type II collagen α1 is specific for cartilaginous tissues, and mutations in its gene are associated with skeletal diseases. Its expression has been shown to be dependent on SOX9, a master transcription factor required for chondrogenesis that binds to an enhancer region in intron 1. However, ChIP sequencing revealed that SOX9 does not strongly bind to intron 1, but rather it binds to intron 6 and a site 30 kb upstream of the transcription start site. Here, we aimed to determine the role of the novel SOX9-binding site in intron 6. We prepared reporter constructs that contain a *Col2a1* promoter, intron 1 with or without intron 6, and the luciferase gene. Although the reporter constructs were not activated by SOX9 alone, the construct that contained both introns 1 and 6 was activated 5–10-fold by the SOX9/SOX5 or the SOX9/SOX6 combination in transient-transfection assays in 293T cells. This enhancement was also observed in rat chondrosarcoma cells that stably expressed the construct. CRISPR/Cas9-induced deletion of intron 6 in RCS cells revealed that a 10-bp region of intron 6 is necessary both for *Col2a1* expression and SOX9 binding. Furthermore, SOX9, but not SOX5, binds to this region as demonstrated in an electrophoretic mobility shift assay, although both SOX9 and SOX5 bind to a larger 325-bp fragment of intron 6 containing this small sequence. These findings suggest a novel mechanism of action of SOX5/6; namely, the SOX9/5/6 combination enhances *Col2a1* transcription through a novel enhancer in intron 6 together with the enhancer in intron 1.

SOX9 is a major transcription factor that regulates chondrocyte differentiation (1–3). The role of SOX9 in chondrogenesis was suggested first by the identification of heterozygous mutations in and around the *SOX9* gene in the human genetic disease campomelic dysplasia, which is a cartilage disease that causes severe skeletal malformations and is often associated with XY female sex reversal (4, 5). Furthermore, site- and stage-specific down-regulation of SOX9 mRNA by mutations in a noncoding region near the *SOX9* gene have been shown to cause Pier-Robinson syndrome, which is characterized by the abnormal positioning of the jaw, cleft palate, and airway obstruction (1).

During chondrocyte differentiation, SOX9 regulates each step, from mesenchymal condensation and chondrocyte differentiation to the formation of hypertrophic chondrocytes (6). Many genes have been shown to be direct targets of this transcription factor, including genes encoding extracellular matrix (ECM)[4] proteins, membrane proteins, other transcription factors, and signaling molecules (7–9). Some of the ECM proteins that are regulated by SOX9 include COL2A1, COL9A2, ACAN, PRELP, and MATN 3.

The expression of one of these ECM proteins, type II collagen α1 (COL2A1) is necessary for chondrocyte differentiation (10). Type II collagen α1 is specific for cartilaginous tissues and necessary for the normal embryonic development of the skeleton. Mutations in this gene cause achondrogenesis, chondrodysplasia, and a loss of tensile strength of bones (11–13).

A SOX9-dependent enhancer was discovered in intron 1 of *Col2a1* gene and has been extensively studied and shown to contribute to *Col2a1* regulation (7–9, 14–20). Other SOX family proteins, such as SOX5 and SOX6, have also been shown to bind to the enhancer fragment and form a large complex with SOX9, although the SOX5 and SOX6 proteins do not have a transactivation domain (17).

Additionally, SOX9 ChIP-Seq and RNA-Seq data from chondrocytes have shown that many chondrogenesis-associated genes are direct targets of SOX9 (7, 8, 21, 22). Furthermore, SOX9 and SOX5/SOX6 have been shown to be located near each other in chromatin (21). However, the detailed regulatory mechanism of SOX9-dependent transcription has not yet been elucidated in relation to the role of SOX5/SOX6 proteins and the enhancers.

Although the expression of *Col2a1* has been thought for >16 years to rely on the SOX9-dependent enhancer in intron 1 (19, 20), we have detected, through a SOX9 ChIP-Seq experiment as shown here, a strong SOX9-binding site in intron 6 but not in intron 1. Therefore, our aim is to elucidate the function of the SOX9-binding site in intron 6.

* This work was supported by the Science Research Center (2015R1A 5A1009701) from the National Research Foundation of Korea, Republic of Korea. The authors declare that they have no conflict of interest with the contents of this article.

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[4] The abbreviations used are: ECM, extracellular matrix; COL2A1, type II collagen α1; RCS, rat chondrosarcoma cell; qPCR, quantitative PCR; EGFP, enhanced GFP; HMG, high-mobility group.

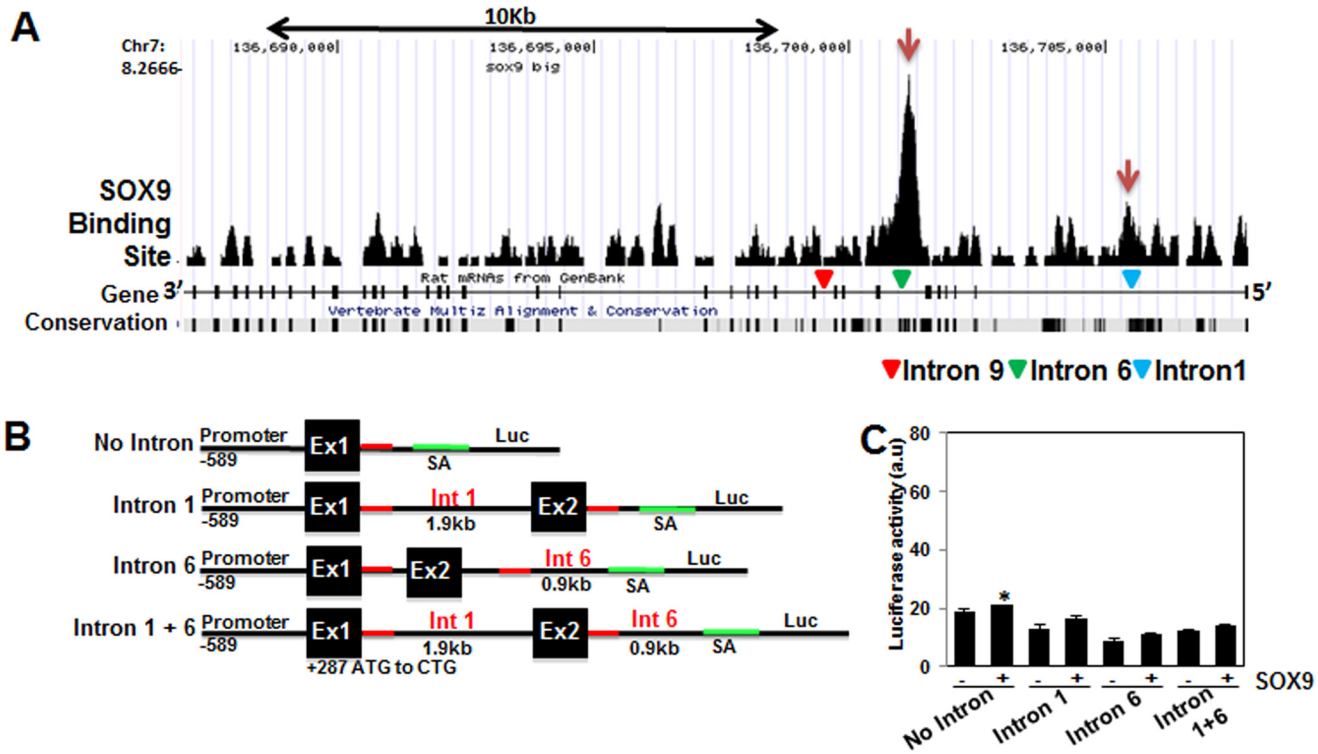

FIGURE 1. **SOX9 ChIP-Seq of *Col2a1* gene and schematic representation of reporter constructs used here.** *A*, the SOX9 interaction sites in *Col2a1* are shown. The *bold boxes* indicate exons. Vertebrate conservation tracks are shown at the *bottom*, and the *bold boxes* indicate highly conserved regions among vertebrate species. The tracks and the conserved regions were obtained from the UCSC Genome Browser using the rat genome assembly (November 2004, Baylor 3.4/rn4). The *arrows* show a strong interaction site in intron 6 and a weaker site in intron 1. *B*, the reporter constructs used in this study. The intron 1 used here was almost a half of intron 1, which contains the well known enhancer sequence. *C*, the luciferase activity of the constructs in the presence of SOX9 alone. The activity is expressed in arbitrary units (*a.u.*). Values are presented as the mean ± S.D. *, $p < 0.01$.

Results

SOX9 Binds to Intron 6 of the Col2a1 Gene—Based on the reported data, we expected that SOX9 would bind to the enhancer region in intron 1, but the ChIP-Seq data of SOX9 in the *Col2a1* gene using rat chondrosarcoma cells (RCS cells) show that there is a strong SOX9-binding site in intron 6 and a weaker binding site in intron 1 (Fig. 1*A*). We also carried out SOX9 ChIP-Seq on mouse primary rib chondrocytes and found that SOX9 bound to intron 7, which corresponds to the rat intron 6 (data not shown) (8). The entire sequence homology of the rat intron 6 to the corresponding mouse sequence (intron 7) or human sequence (intron 7) is 90 and 85%, respectively, and the SOX9-binding sites seemed to correspond to the highly conserved locus deduced from the *Col2a1* genes of six other vertebrates retrieved from the UCSC Genome Browser (Fig. 1*A*, *bottom*). We then attempted to determine the role of the SOX9-binding region in intron 6.

We first tested whether intron 6 has SOX9-dependent enhancer activity. We prepared several constructs containing no introns, intron 1 alone, intron 6 alone, or both introns 1 and 6 (shown in Fig. 1*B*). These four constructs contained the 589-bp *Col2a1* minimum promoter and exon 1. Intron 1 contains a DNA sequence that was previously reported to be a SOX9-dependent enhancer (19, 20). However, we found that the reporter activity in any of these constructs was not significantly enhanced by SOX9 (Fig. 1*C*). As reported elsewhere (9, 16), a construct with four or five tandemly repeated SOX9-binding sites in intron 1 showed a high SOX9-dependent enhancer activity with the minimum *Col2a1* promoter. The strong enhancer activity was observed only when the tandem repeats were used. Thus, these results suggested that the constructs with a single binding fragment cannot exert enhancer action in a transient reporter assay in 293T cells.

The Effect of SOX5/SOX6 on the Enhancer Activity of Introns 1 and 6—We then evaluated the effect of SOX5 on the enhancer activity in these constructs (Fig. 2*A*). SOX5 enhanced the reporter activity in each construct 2-fold at most. In the presence of both SOX9 and SOX5, the reporter activities of the constructs with only the promoter, the promoter plus intron 1, or the promoter plus intron 6 showed at most 3-fold enhancement. Although the construct containing both introns 1 and 6 showed ~6-fold enhancement of reporter activity in the presence of both SOX9 and SOX5, in the presence of only one of the two SOX proteins, the enhancement was less than 2-fold. These data strongly suggest that the presence of both introns 1 and 6 has a strong enhancer activity that is dependent on the combination of SOX9 and SOX5. The protein expression of SOX5 and SOX9 in this system has been confirmed by Western blotting of each protein (Fig. 2*B*).

Next, the importance of SOX5 DNA binding activity for the enhancer activity was assessed. SOX5 has a DNA binding domain (the HMG domain) spanning amino acid residues 474 and 553 (Fig. 2*C*). The deletion mutant of the DNA binding domain of SOX5 did not show any enhancer activity (Fig. 2*D*). In this experiment we also tested the effect of combining SOX9 and SOX6 on the enhancement of the luciferase activity. As shown in Fig. 2*D*, the combination of SOX9 and SOX6 had an

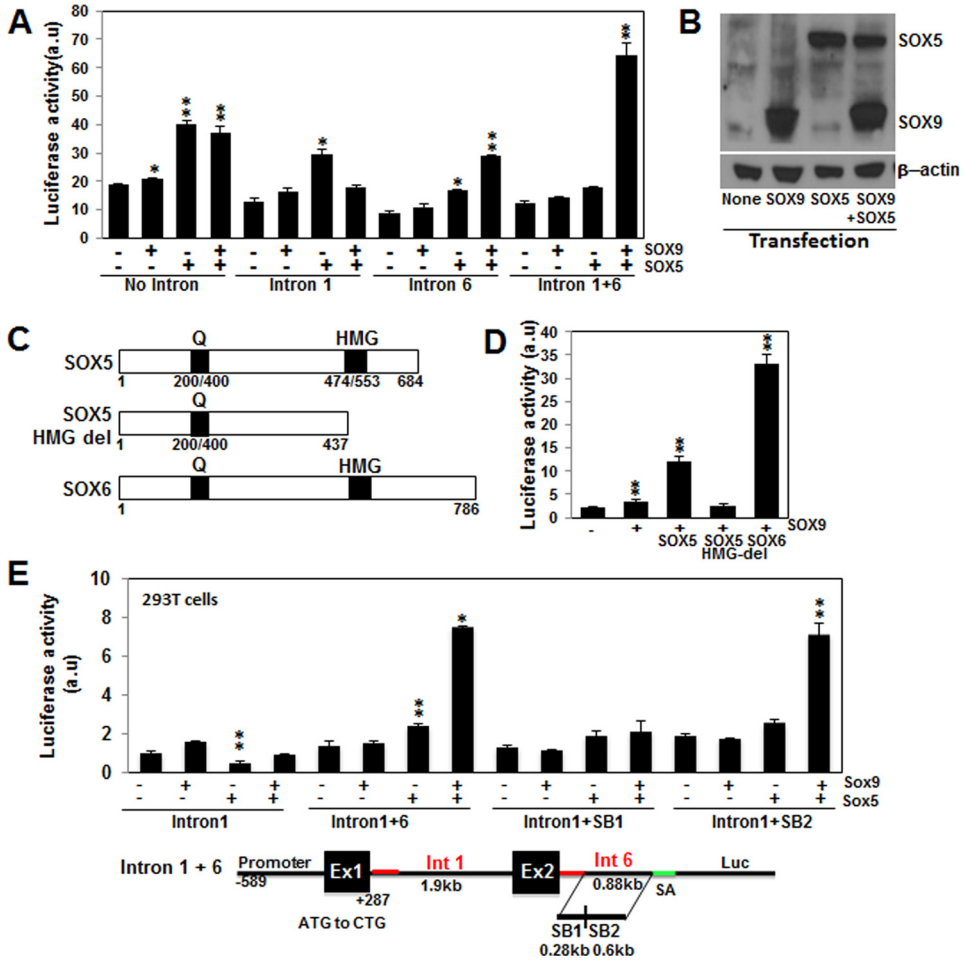

FIGURE 2. **Enhancement of luciferase activity using various constructs in the presence of SOX9 and/or SOX5.** *A*, luciferase activity of the constructs shown in Fig. 1*B* in the presence of SOX9 alone, SOX5 alone, and both SOX9 and SOX5. *B*, Western blotting of ectopically expressed SOX9 and SOX5 in the reporter assay. *C*, structures of SOX5 and SOX6. The HMG and the Q domain are a DNA binding domain and a glutamic acid-rich domain, respectively. *D*, luciferase reporter activity in the presence of the indicated SOX protein(s). *E*, effect of the dissection of intron 6 on the reporter activity. Intron 6 (0.88 kb) was dissected into SB1 (0.28 kb) and SB2 (0.6 kb). Values are presented as the mean ± S.D. * and ** show $p < 0.01$ and $p < 0.05$, respectively. *a.u.*, arbitrary units.

even stronger activity than the combination of SOX9 and SOX5. SOX5 and SOX6 have been shown to compensate for each other's activity in experiments on knock-out mice (23). Our data also seem to support this notion.

We then focused on the location of SOX9 interaction sites in intron 6. Intron 6 (0.88 kb) was divided into two portions, SB1 (5′ end, 0.28 kb) and SB2 (3′ end, 0.6 kb) (Fig. 2*E*). In 293T cells, the construct with SB1 showed no SOX-dependent activity, whereas the construct with SB2 did (Fig. 2*E*).

Because the SOX9 ChIP-Seq data also revealed the existence of SOX9-binding sites 30 kb upstream of the *Col2a1* transcription initiation site (Fig. 3*A*), we tested whether the strong binding site has characteristics similar to those of intron 6 with respect to SOX9-dependent enhancer activity. When the site was ligated to the 5′ end of the same reporter construct used in Fig. 2, the resulting construct was 1.5-fold more strongly activated in the presence of both SOX9 and SOX5 than the construct without the site (Fig. 3*B*).

To confirm the enhancer activity of introns 1 and 6 in cultured cells, the constructs with intron 1 only or with both introns 1 and 6 (Fig. 4*A*) were transfected into RCS cells, whose endogenous expression levels of SOX9, SOX5, and SOX6 are very similar to those of chondrocytes (9, 18, 31), and the colonies that stably expressed the construct (*i.e.* they were resistant to G418 at a final concentration of 0.5 mg/ml) were chosen. All the colonies that formed in a dish were pooled, and the total luciferase activity was measured. Using this assay, the number of constructs inserted into the genome and the location of the insertion could be normalized. In agreement with the data from the transient-transfection assay, the construct containing both introns 1 and 6 had much higher activity than the construct containing intron 1 alone (Fig. 4*B*). Similar data were obtained with cells cultured at higher G418 concentration (0.75 mg/ml) (data not shown).

The above results suggested that both SOX9 and SOX5/SOX6 bind to intron 6 and that the binding of SOX5/SOX6 to intron 6 may facilitate the binding of SOX9 to the intron 1. To explore this possibility, we tested whether SOX9 and SOX5 bound to the SB2 fragment (600 bp) shown above (Fig. 2*E*) or an even shorter fragment by means of an EMSA. As shown in Fig. 4*C*, both SOX9 and SOX5 were found to bind to a 325-bp fragment of intron 6 that is a part of the SB2 fragment. Because several bands were detected in SOX9 EMSAs, monomers and dimers of SOX9 seem to bind to this fragment (15). In contrast,

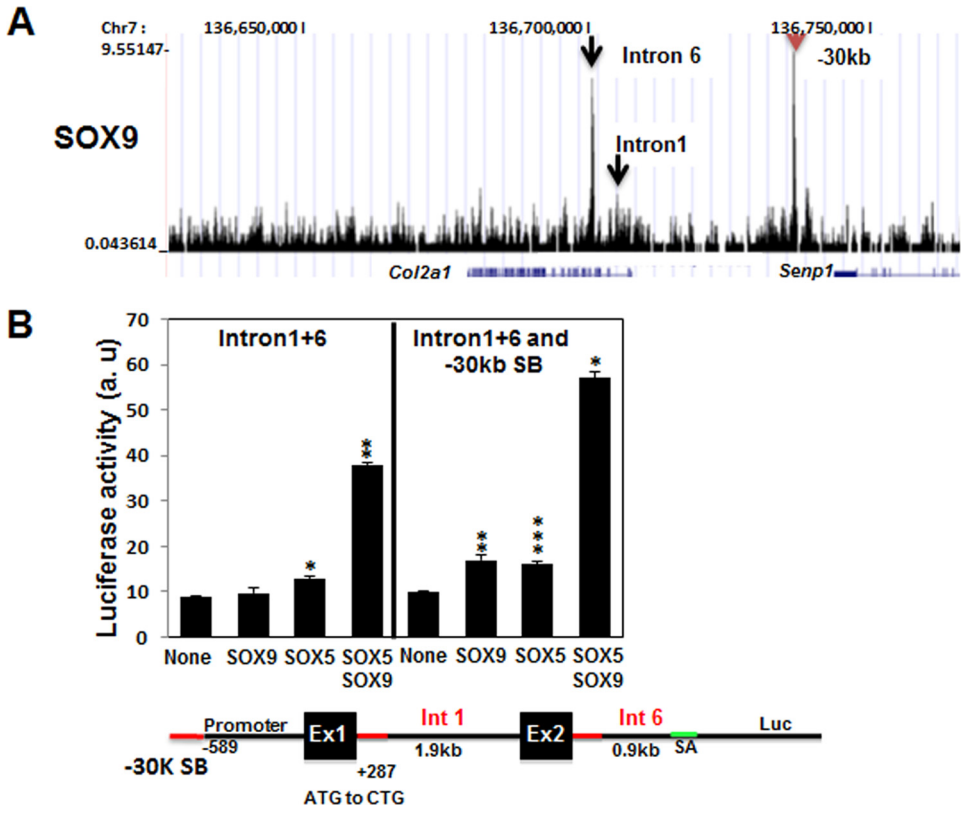

FIGURE 3. **Enhancement of luciferase activity in the construct including a strong SOX9-binding site near −30 kb.** *A*, the SOX9 interaction sites far upstream region of *Col2a1* gene analyzed by SOX9 ChIP-Seq. The direction of this gene is from *right* to *left*. A strong binding site (*red arrow*) is located ~30 kb upstream from the transcription initiation site. *B*, the DNA fragment including the SOX9-binding site near position −30 kb was ligated upstream of the promoter in the reporter construct, which has both introns 1 and 6 (Fig. 1*B*). The reporter activities were measured under various conditions shown *below each histogram*. Values are presented as the mean ± S.D. *, **, and *** show $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively. *a.u.*, arbitrary units.

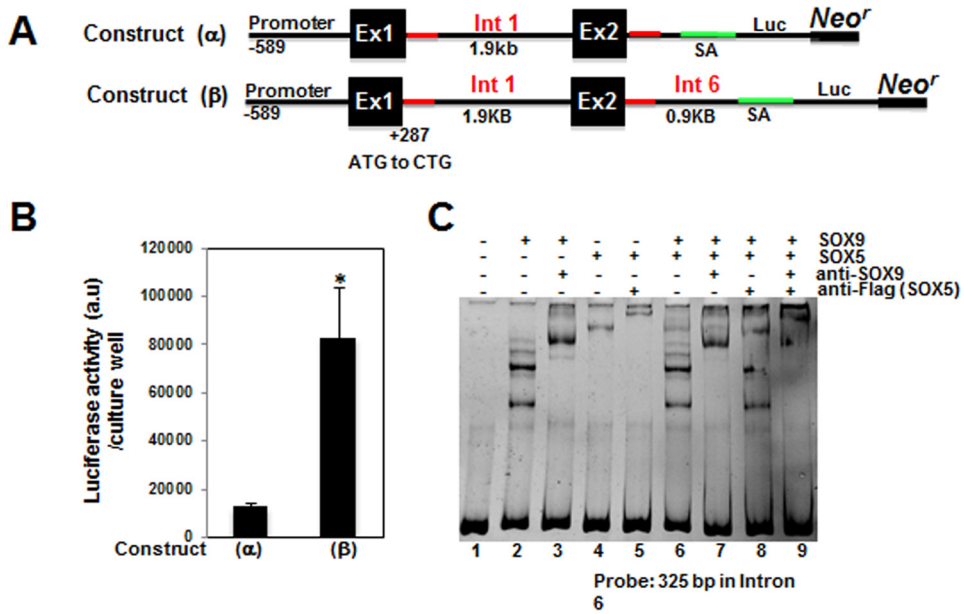

FIGURE 4. **The reporter activity of RCS cells stably expressing constructs with either intron 1 alone or both introns 1 and 6 and the evidence of binding of SOX9 and SOX5 to intron 6.** *A*, the constructs transfected into RCS cells. Construct α does not contain intron 6 but construct β does. Both constructs have neomycin resistance genes driven by an SV40 minimum promoter. *B*, reporter activity in cells stably expressing each construct in the presence of 0.5 mg/ml G418. Six independent plates for each construct were used for these experiments. Values are presented as the mean ± S.D. *, $p < 0.0005$. *C*, the EMSA was performed using the 325-bp fragment of SB2 region of intron 6 in Fig. 2*E* as a probe. Each binding protein was shown *above each lane*. In *lanes 3*, 5, 7, 8, and 9, the super-shifted SOX9 and/or SOX5/probe/antibody complexes are observed. *a.u.*, arbitrary units.

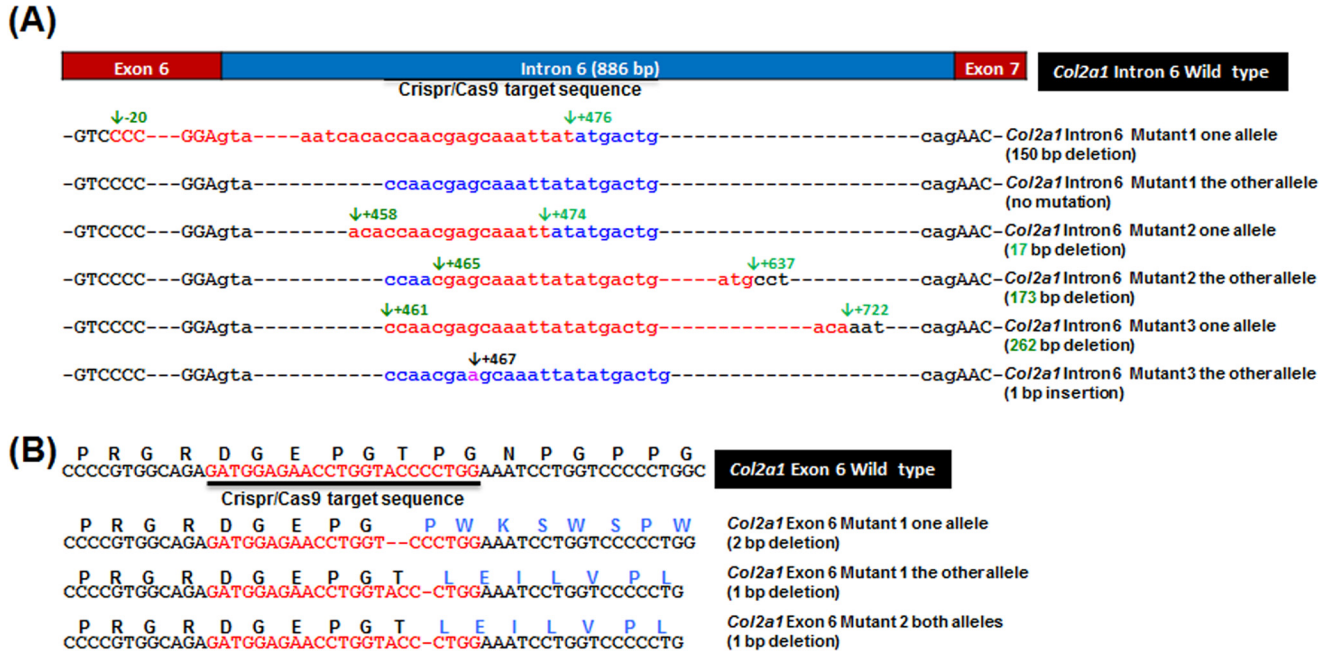

FIGURE 5. **Mutations found in intron 6 and exon 6 mutants.** *A*, deletions in the intron 6 mutants, mutants 1, 2, and 3 (Int6-M1, -M2, and -M3). In the *top*, the *Col2a1* wild-type sequence is shown. The bases in exons and intron are shown as *uppercase* and *lowercase*, respectively. The Crispr/Cas9 target sequence is shown in *blue*, and the deleted or inserted base(s) in each allele in each mutant is shown in *red*. The first base of intron 6 is shown as +1, and the bases upstream (in exon 6) are shown from −1 through −23. The base at the end of 5′ or 3′ of deleted sequence is numbered in *green*. The inserted base in Mutant-3 (int6-M3) is numbered in *black*. *B*, deletions in the exon 6 mutants, mutant 1 and 2 (ex6-M1 and -M2). In the *top*, *Col2a1* wild sequence is shown. The CRISPR/Cas9 target sequence is shown in *red*, and the deleted bases are shown with a *dashed line* in each allele in each mutant. The amino acid sequence of partial 3′ region from deleted base(s) is deduced from DNA sequence and shown in *blue*.

in the case of SOX5, only one band was detected, which may be the dimer of SOX5 (Fig. 4*C*, *lane 4*) (17). The molecular mass of FLAG-SOX5 is ~80 kDa, which is much greater than the 50 kDa of SOX9, and the shifted band with FLAG-SOX5 migrated much more slowly than the band corresponding to SOX9 (Fig. 4*C*, *lanes 2* and *4*). This binding pattern was confirmed with the super-shifted band in the presence of a specific antibody against each SOX protein (Fig. 4*C*, *lanes 3* and *5*). The super-shifted SOX5 band was seen near the top edge of the gel (Fig. 4*C*, *lane 5*). When both SOX9 and SOX5 were combined in the presence of either the anti-SOX9 (Fig. 4*C*, *lane 7*) or anti-FLAG (SOX5) antibody (Fig. 4*C*, *lane 8*), the intensity of each super-shifted band at the top of the gel was stronger than the intensity of the reaction with SOX9 and anti-SOX9 antibody (Fig. 4*C*, *lane 3*) or with SOX5 and the anti-FLAG (SOX5) antibody (Fig. 4*C*, *lane 5*), respectively. This result indicated that at least to some extent both SOX9 and SOX5 bind together to the same fragment.

Confirmation of Enhancer Activity of Intron 6 Using Deletion Mutants—To confirm the importance of intron 6 for the expression of *Col2a1*, we used intron 6 deletion mutants prepared from RCS cells. In addition, we used the deletion mutants in exon 6 as a control of *Col2a1* knock-out. We obtained three intron 6-mutant cell lines, designated int6-M1, int6-M2, and int6-M3. Fig. 5*A* shows the schematic representation of the indel in each mutant. One allele of int6-M1 has a 150-bp deletion from the 3′ region of exon 6 to the middle region of intron 6, and the other allele is wild type. One allele of int6-M2 has a 17-bp deletion in the middle of intron 6, and the other allele has a 173-bp deletion from the middle region of intron 6 to the base pair located 250 bp away from the end of intron 6. One allele of int6-M3 has a 262-bp deletion from the middle region of intron 6 to the base pair located 165 bp away from the end of intron 6, and the other allele has only 1 base insertion in the middle of intron 6.

As a knock-out control of the *Col2a1* gene, two cell lines with deletions in exon 6, ex6-M1 and ex6-M2, were used. Both mutants were found to have frameshift mutations. One allele in ex6-M1 has a 2-bp deletion, whereas the other allele has a 1-bp deletion in exon 6. The ex6-M2 mutant has the same 1-bp deletion in both alleles, and due to this frameshift, these mutants were hypothesized not to express full-length COL2A1 proteins because the deduced amino acid sequences were changed after the deletions (Fig. 5*B*).

First, we examined the mRNA expression levels of *Col2a1*, *Sox9*, *Col9a2*, and *Acan*. In addition to *Col2a1*, *Col9a2* and *Acan* are known to be direct targets of SOX9 (Fig. 6). The mRNA expression of *Col2a1* was quantified by reverse transcription-quantitative PCR (RT-qPCR) using the following two primer sets: one corresponding to exons 5 and 6 and another corresponding to exons 47 and 49 (Table 1). The decrease in *Col2a1* mRNA levels in int6-M2 and ex6-M2 mutants was detected effectively by means of both primer sets. In the case of int6-M1, int6-M3, and ex6-M1 mutants, the decrease in *Col2a1* mRNA level was detected only by means of the primer set corresponding to exons 47–49, and the magnitude of the decrease was close to 40% in the case of int6-M1 and int6-M3 mutants (Fig. 6*A*). The mRNA level of *Sox9* was 1.2–1.5-fold higher in int6-M1, -M2, and -M3 (Fig. 6*A*) and was ~4-fold higher in ex6-M1 and ex6-M2 mutants compared with that in wild-type cells (Fig. 6*B*). The level of *Acan* mRNA increased 1.5- or 3.0-fold in int6-M1 and int6-M2, respectively. The level of mRNA

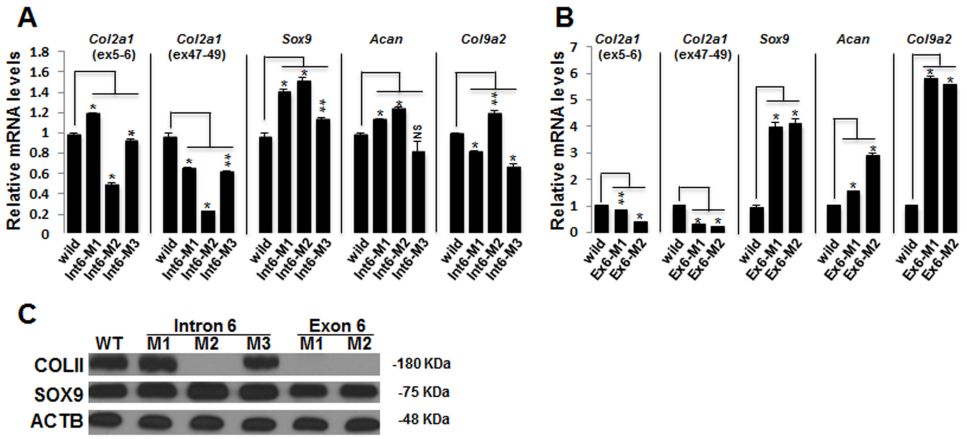

FIGURE 6. **Decreased mRNA and protein expression in intron 6 and exon 6 mutant cells.** *A* and *B*, the mRNA expression of *Col2a1*, *Sox9*, *Acan*, and *Col9a2*. The expression level was measured by qPCR using primers specific for each gene (Table 1). In the case of *Col2a1* mRNA, two primer sets were used; one covers exon 5 to exon 6, and the other covers exon 47 to exon 49. The values are normalized to *Gapdh* expression and compared with each gene expression of wild-type cells. Values are presented as the mean ± S.D. * and ** show $p < 0.001$ and $p < 0.005$, respectively. *A*, the mRNA expression in intron 6 mutants, int6-M1, -M2, and -M3. *NS*, not significant. *B*, the mRNA expression in exon 6 mutants, ex6-M1 and -M2. *C*, Western blotting of COL2A1, SOX9, and β-actin (*ACTB*, loading control).

TABLE 1
List of primers used in this study

ChIP-qPCR	
Col2a1 intron 6 forward	cccgtcgtgcggttaatt
Col2a1 intron 6 reverse	actgctcttccagagaaacacaagt
Col2a1 intron 9 forward	gcaacagggagtgccatagg
Col2a1 intron 9 reverse	agctcctgtgatattacttctctgcaa
Col9a2 forward	cgtgaagcacgtctccttgg
Col9a2 reverse	cagacagtacacagacagaactggg
qPCR for mRNA quantification	
Col2a1 exon 5–6 forward	gacctgccggtgaacaag
Col2a1 exon 5–6 reverse	ggtaccaggttctccatctct
Col2a1 exon 47–49 forward	gacaaaggagaagctggagag
Col2a1 exon 47–49 reverse	ttagagccatctttgccagag
Sox9 forward	ctctcctaacgccatcttcaag
Sox9 reverse	acgtctgttttgggagtgg
Acan forward	cagaagggtcaggagaaacag
Acan reverse	gcatgtgaaagtgtccaagg
Col9a2 forward	cctgaagacaagttcagtgagg
Col9a2 reverse	gctgaacagcatgggtaaga
Cy3-labeled EMSA probe	
rat *Col2a1* intron 1 sense	cactcgagaaaagccccattcatgagagg
rat *Col2a1* intron 6 sense	ctgtcgtgtttggaatcacaccaacgagca
mouse *Col2a1* intron 6 sense	ctgtcgtgtttggaatcacaccagtcaaca
Cy3-labeled Primers for EMSA probe made by PCR	
Col2a1 intron 6 forward	gaaggtgtgtggctactgtcg
Col2a1 intron 6 reverse	tcgcttccttgggaagtcaag
Primers for PCR to detect mutation	
For intron 6-mutants *Col2a1* intron 6 forward	ggttcttaaggaagcattgcttcgctgatacc
For intron 6-mutants *Col2a1* intron 6 reverse	ctgcaaagcaacacaactgggttagg
For exon 6-mutant *Col2a1* exon 4 forward	atcataggacctaaaggacctcctgg
For exon 6-mutant Col2a1 intron 6 reverse	gcctcgagcctcatgtgtgattctcctc
Primers for preparing *Col2a1* exon6/intron6/ exon7-EGFP	
Col2a1 exon6 forward	ggcttccttagactcttctttgggag
Col2a1 exon7 reverse	gacttgaggtgaaacccatgaacagg

of *Acan* in ex6-M1 or ex6-M2 mutants was increased to 1.6- or 2.9-fold the level in wild-type cells. *Col9a1* expression was increased ~6-fold in ex6-M1, and ex6-M2 mutants were compared with that in wild-type cells.

Next, we analyzed the expression levels of two proteins, type II collagen α1 (COL2A1) and SOX9, in each cell clone. The level of β-actin served as a loading control. The amount of COL2A1 decreased markedly in int6-M2, ex6-M1, and ex6-M2 mutants, and the protein was hardly detectable (Fig. 6*C*). In int6-M1 and int6-M3 cells, COL2A1 was not decreased compared with that in wild-type cells even though its mRNA levels were decreased in these mutants. The protein level of SOX9 did not significantly change in all mutant cells, including ex6-M1 and ex6-M2 mutants, where its mRNA levels were >4-fold higher than that in wild-type cells.

The Deletion Mutants Did Not Have Splicing Defects—Furthermore, we demonstrated that the decrease in *Col2a1* expression is not due to perturbation of splicing intron 6 in the mutants. To test whether the various lengths of intron 6 affected splicing, we constructed five splicing reporter plasmids, as shown in Fig. 7*A*. Each construct contained exons 6 and 7 followed by an EGFP gene, an initiation codon (ATG)

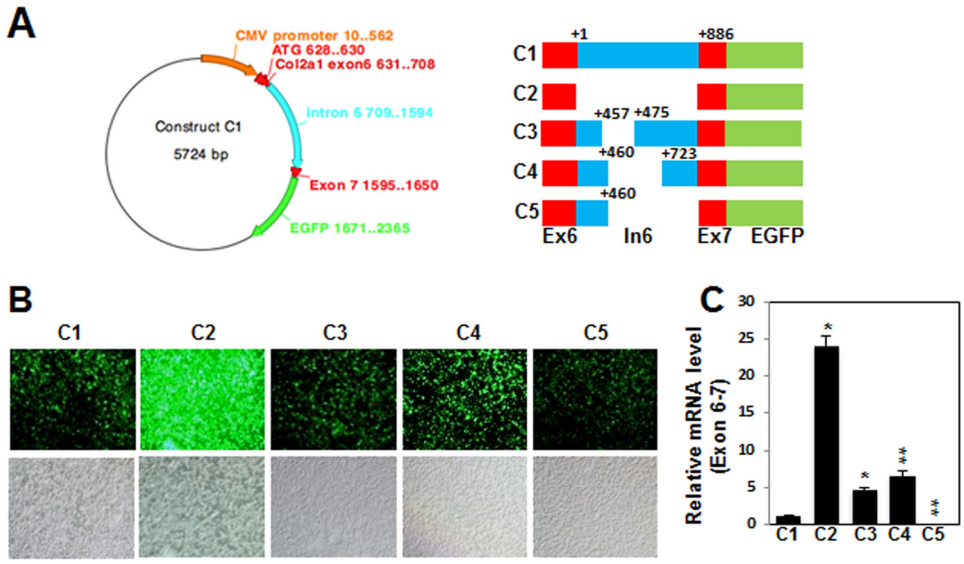

FIGURE 7. **Effects of deletion of intron 6 on mRNA splicing.** *A*, *left*, a map of the pEGFP-construct (*C1*). The ATG plus partial exon 6, entire intron 6, and partial exon 7 of *Col2a1* were ligated to the 5′ end of the EGFP gene. Exon 7 and EGFP are in-frame. This construct was made from pEGFP-N1 vector (Clontech), and the transcription is under the control of the CMV promoter. *Right*, schematic illustration of the constructs used. In each construct, C1 through C5, the length of intron 6 varies. *B*, the expression of EGFP in cells transfected with each construct. The *upper* and *lower panels* show the EGFP expression and the same view by bright-field microscopy, respectively. *C*, the expression of mRNA of exon 6 through exon 7. The level of mRNA expression was measured by RT-qPCR using the primer pair amplifying exon 6 and exon 7 (Table 1). The values are normalized to *Gapdh* expression in each cell clone transfected, and the expression levels were calculated by the $\Delta\Delta C_t$ method with construct C1 as a control. Values are presented as the mean ± S.D. * and ** show $p < 0.001$ and $p < 0.005$, respectively.

before the exon 6, and differing lengths of intron 6 between the two exons (Fig. 7*A*). The *EGFP* gene is designed to be in-frame with exon 7, and therefore, if intron 6 is spliced properly, then EGFP expression should occur. When each construct is transfected to 293T cells, the expression of EGFP was the strongest in construct C2 (without intron 6) (Fig. 7*B*, *C2*). The constructs (constructs C3 and C4) that have a partial deletion of intron 6 showed higher expression of EGFP than did the construct with the entire intron 6 (construct C1) (Fig. 7*B*). Construct C5 (with neither the splicing branch residue nor 3′ splice site at the intron 6-exon 7 junction) showed the weakest signal of EGFP (Fig. 7*B*, *C5*). This weak signal is likely to be due to the expression of mRNA starting from ATG of the EGFP gene, taking into account the analysis of the size of this mRNA (data not shown). Next, we confirmed these phenomena by analyzing the expression level of correctly spliced mRNA. The total RNA was purified from each transfected cell clone, and quantitative RT-PCR (RT-qPCR) was performed. *Gapdh* mRNA expression was used as an RT-PCR assay control. The constructs with the partial deletion of intron 6 (Fig. 7*C*, *C3* and *C4*) resulted in a higher level of spliced mRNA expression than that corresponding to the complete intron 6 (Fig. 7*C*, *C1*). The expression levels of the 17-bp deletion construct C3 (corresponding to int6-M2 short deletion-allele) and of the 262-bp deletion construct C4 (corresponding to int6-M3 allele and also including int6-M2 long deletion-allele) were >4-fold and >6-fold higher than that of the construct that contained full intron 6, respectively. The construct C2 without intron 6 showed almost 22-fold higher expression than C1. The construct C5, which lacks a branch site and splicing acceptor, expressed longer mRNA, indicating that it was not spliced or not spliced correctly (data not shown). These data clearly show that the decrease in *Col2a1* expression observed in int6-M2 and int6-M3 mutants was not due to a splicing defect.

SOX9-binding Site in Intron 6—To confirm that SOX9 does not bind to, or binds only weakly to intron 6 in the int6-M2 mutant, we performed SOX9 ChIP-qPCR using this mutant and wild-type cells. As the positive control of SOX9 ChIP-qPCR, intron 1 of *Col9a2* gene was used, because a SOX9-binding site was confirmed in intron 1 of *Col9a2* by SOX9-ChIP-Seq (Fig. 8*A*). In intron 6 of *Col2a1* gene, the qPCR probe was designed near the deletion site of the int6-M2 mutant (Fig. 8*B*, Table 1). As shown in Fig. 8*C*, SOX9 bound to intron 6 in wild-type cells and hardly bound to intron 6 in the int6-M2 mutant. As expected, SOX9 bound to intron 1 of *Col9a2* in both wild-type and int6-M2 mutant cells (Fig. 8*C*). In contrast, minimal binding of SOX9 to intron 9 of the *Col2a1* gene was observed in both the int6-M2 mutant and wild type (Fig. 8*C*). This result was consistent with the finding that a SOX9-binding site was not detected in intron 9 of the *Col2a1* gene by SOX9 ChIP-Seq (Fig. 1*A*).

We then performed an EMSA to determine whether SOX9 binds to this region *in vitro*. First, we assessed whether the nuclear extract from RCS cells could bind to the 30-bp fragment in intron 6 (Fig. 8*H*, Table 1), which includes the 13-bp sequence of the 17-bp deleted in int6-M2 mutant cells. As shown in Fig. 8*D*, *left panel*, the labeled probe was shifted, and the intensity of the band was decreased as the result of the competition with the non-labeled probe. A similar EMSA pattern was observed using the well known enhancer sequence in intron 1 (Table 1). The rat sequence has high homology with the corresponding sequences of mouse and human (Fig. 8*H*), and the nuclear extract from mouse rib chondrocytes was also shown to bind to the mouse sequence (Fig. 8*D*, *right panel*). Then, purified SOX9 was applied to the EMSA and was shown to bind the rat and mouse probes used (Fig. 8, *E* and *F*). Its binding was confirmed by the super-shifted bands in the pres-

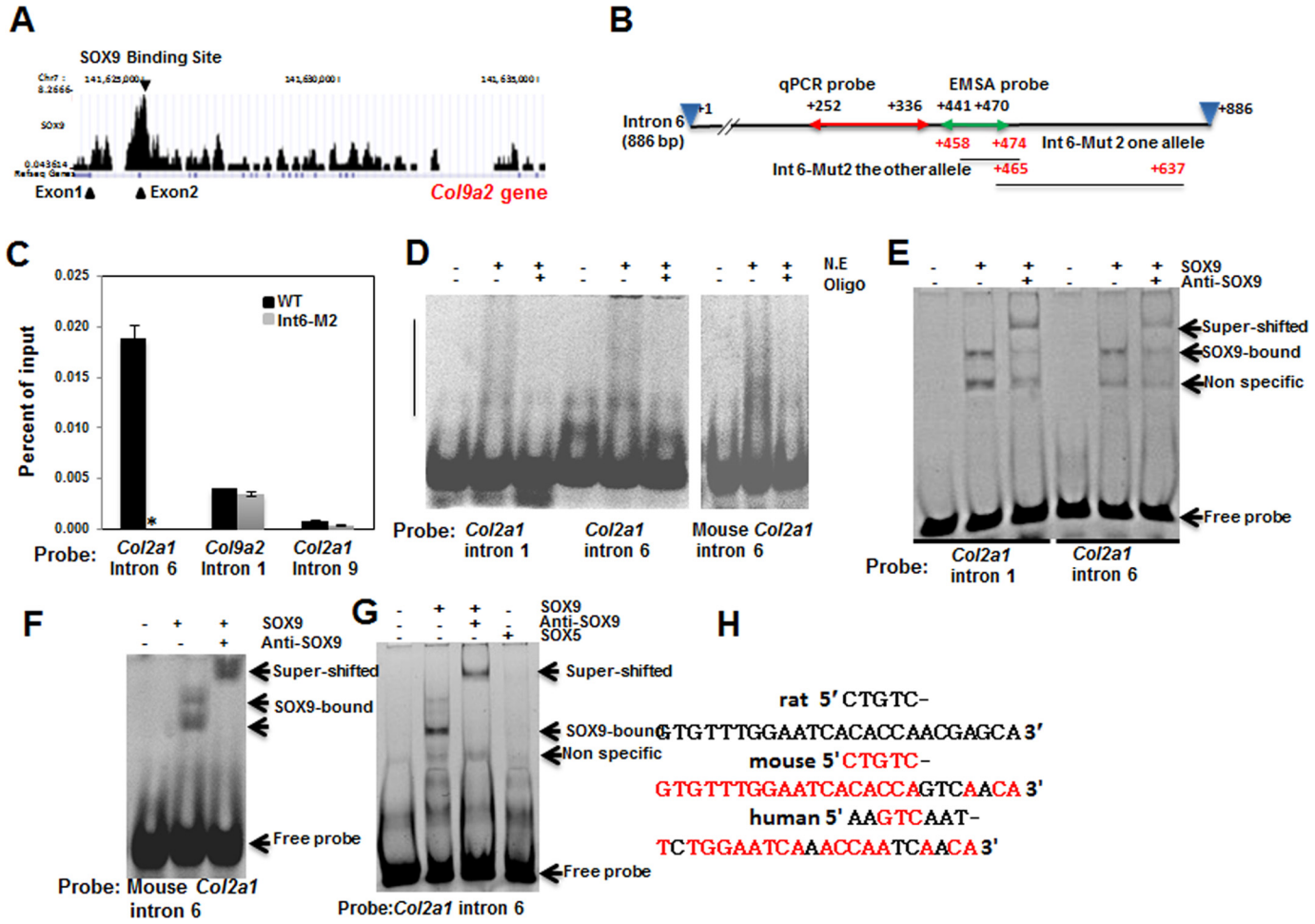

FIGURE 8. **A SOX9-binding site in intron 6 of *Col2a1*.** *A*, the SOX9 interaction site in *Col9a2* revealed by SOX9-ChIP-Seq. The *arrow* shows the strong binding site in the region spanning from intron 1 through exon 2. *B*, the deleted regions of the two alleles of the *Col2a1* gene and the probes used for ChIP-qPCR and EMSA. The detailed deleted sequence and sequences of the probes are shown in Fig. 5 and Table 1, respectively. *C*, SOX9 ChIP-qPCR using wild-type cells and int6-M2 mutant cells. Values are presented as the mean ± S.D. *, $p < 0.001$. *D*, EMSA was performed using the proposed rat or mouse SOX9-binding site shown in *H*. The well characterized SOX9-binding site in rat intron 1 was used as a control. The nuclear extract of RCS cells or mouse rib chondrocytes was used in the *left* or *right panel*, respectively. The sifted bands are detected in the region shown by *vertical bar*. *E*, the EMSA using purified SOX9 protein and rat intron 6 probe. *F*, EMSA using purified SOX9 and mouse intron 6 probe. *G*, EMSA using purified SOX9 or SOX5 and rat intron 6 probe. *H*, alignment of the proposed rat SOX9-binding site (EMSA probe) to the corresponding site in mouse or human. The bases with shared homology to the rat sequence are shown in *red*.

ence of anti-SOX9 antibody (Fig. 8, *E* and *F*). In a parallel EMSA, SOX5 did not bind to this sequence (Fig. 8*G*).

Discussion

In this study we report two main observations. First, a novel SOX9-binding site in intron 6 seems to be necessary for the strong expression of *Col2a1*. Second, this enhancer activity depends on the combination of SOX9 and either SOX5 or SOX6.

The existence of an enhancer in intron 6 was previously proposed by Shinomura *et al.* (24), who reported enhancer-like activity in intron 6 in RCS cells, although the proposed SOX9 binding sequence is different from the one shown here. They detected the enhancer-like activity by an assay based on the co-transfection of the *Col2a1* reporter plasmid containing intron 1 and intron 6 fragments. However, a biochemical assay was not demonstrated to confirm that the activity is dependent on the presence of SOX9. Therefore, it was unclear whether the enhancer activity depended on SOX9 or another factor(s).

The importance of the SOX9-binding site in intron 6 was revealed in the mutant RCS cells. Especially in the int6-M2 mutant, which has deletions in both alleles of the *Col2a1* gene, a marked decrease in *Col2a1* expression was observed both at mRNA and protein levels. By Sox9-ChIP-qPCR, we clearly showed that SOX9 does not bind intron 6 in this mutant. Both alleles in this mutant lack the same 10-bp region. Using EMSA, SOX9 was shown to bind to the region including a part of this region, whereas SOX5 hardly binds to this SOX9-binding site. The binding site shows a hairpin structure, but does not have the same motif that was recently demonstrated by ChIP-Seq to be a canonical SOX9-binding inverted repeat motif (7, 8, 21, 22). The proposed rat SOX9-binding site has high homology to the corresponding site in the mouse and human sequence, and the mouse sequence also binds to SOX9. Furthermore, we showed by EMSA that both SOX9 and SOX5 bind to a 325-bp DNA fragment, which includes the 10-bp sequence shown above. In a recent study, Liu and Lefebvre (21) stated that SOX5

or SOX6 has a binding site near the SOX9-binding site, and the binding site seemed to contain a direct, not inverted repeat of the SOX binding motif. However, we could not identify where the SOX5 or SOX6 binding site in intron 6 is, and its exact binding site should be clarified in future studies.

The experiments involving mutant cells generated using the CRISPR/Cas9 system have yielded useful information regarding the existence of the SOX9-binding site in intron 6, which is indispensable for high expression of the *Col2a1* gene, as discussed above. In the experiments using mutant cells, we ruled out the possibility that a partial deletion of intron 6 in the mutants decreased the mRNA expression due to a splicing defect. In the case of the int6-M1 mutant, an intron splicing donor site in exon 6 of one allele was deleted. This deletion caused the decrease in the level of *Col2a1* mRNA but did not alter the expression level of the COL2A1 protein, which was similar to int6-M3. One possible reason is that the mRNA transcribed from the other alleles of the mutants might compensate for the amount of protein, but the exact reason has been remained to be clarified.

In addition, several unexpected phenotypes have been observed in the mutant cells. The experiments clearly showed that int6-M2, ex6-M1, and ex6-M2 mutants have a markedly decreased expression level of *Col2a1* mRNA and COL2A1 protein expression. However, in the *Sox9* gene, the parallel relation between mRNA and protein expression is not clear. The *Sox9* mRNA levels in ex6-M1 and ex6-M2 mutants are increased 4-fold, but the SOX9 protein expression is not increased. These phenomena may be explained in part by the degradation of SOX9 by the ubiquitin-proteasome system and/or translational inhibition by microRNAs (25, 26). In addition, it remains to be elucidated why the mRNA expression level of its targets, *Col9a2* and *Acan*, increased so much in the mutant cells where the *Col2a1* gene is disrupted. We do not think this was caused by an off-target effect of the CRISPR/Cas9 system used here, and further experiments are necessary to elucidate the phenomena.

Several transcription regulators that bind to SOX9 have been identified, and the involvement of these regulators in the expression of *Col2a1* gene together with SOX9 have been discussed. These proteins include TIP60 (27), p300/CBP (28), ZNF219 (29), Wwp2, Med25 (30), and PIAS (31). Because SOX9-binding sites exist in not only intron 1 but also in intron 6 and ~30 kb upstream of the gene, these regulators may form a large complex with SOX9 and SOX5/SOX6, spanning from the first SOX-binding site in the region 30 kb upstream of the gene to intron 6, connecting the complex to the basal transcription machinery, including polymerase II (Fig. 9).

The sequence of intron 6 and intron 1 has a high homology among rats, mice, and humans. Although defects in type II collagen expression cause several diseases, to our knowledge there have been no reports of a disease-related mutation in intron 6 and intron 1. Extensive analysis of these introns in the genomes of patients with type II collagen-related diseases would be worthwhile.

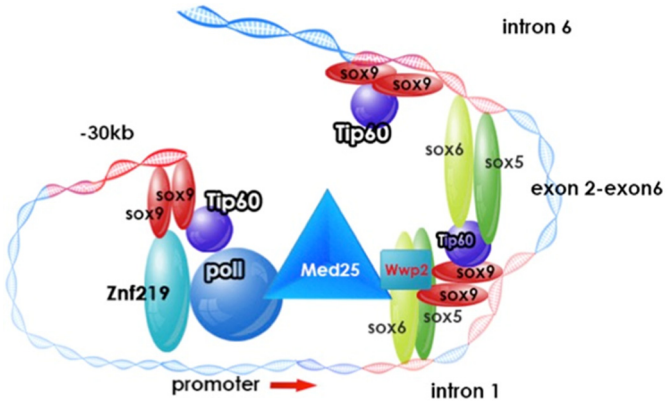

FIGURE 9. **The putative transcription machinery of the *Col2a1* gene.** Our results suggest that SOX9 and SOX5/SOX6 bind to intron 1, intron 6, and at the site located in the −30-kb region. SOX5 and SOX6 may cause the DNA to bend as previously shown (23). Many other factors, including TIP60, ZNF219, WWP2, and MED25, have also been shown to bind to SOX9. MED25 has also been shown to bind to RNA polymerase II.

Experimental Procedures

Cell Culture, Plasmid Transfection, and a Reporter Assay—Human 293T cells and rat chondrosarcoma cells (RCS cells) were cultured in DMEM supplemented with 10% fetal bovine serum. 293T cells were transfected with a plasmid using either FuGENE 6 (Promega) in a reporter assay or by the calcium phosphate method in the experiment to generate the FLAG-SOX5 protein. To prepare stable cell colonies of RCS cells, the cells were transfected with a plasmid using Lipofectamine 2000 transfection reagent (Invitrogen), and the colonies stably expressing the gene of interest were selected in the presence of G418 at a final concentration of 0.5 or 0.75 mg/ml. The reporter Firefly luciferase plasmid, other appropriate plasmid(s), and a CMV-*Renilla* luciferase plasmid were co-transfected into the cells. The CMV-*Renilla* luciferase plasmid served as a transfection control, and the Firefly luciferase expression level was normalized to the expression of *Renilla* luciferase in the same sample using a dual-luciferase assay system (Promega) as previously shown (9).

Plasmid Construction—The construct was created from the plasmid that contains the mouse *Col2a1* promoter, exon 1 (the first ATG is mutated to CTG), the splicing acceptor site, and *β*-geo as previously reported (18). DNA fragments from the mouse *Col2a1* gene that were used to construct plasmids were obtained by PCR amplification using primers and the RP23-345G Bac clone (BacPac Resources) as the template as described elsewhere (9). The plasmids carrying human *SOX9*, *SOX6*, *SOX5*, or *SOX5* mutants have been previously described (9, 17, 32). The constructs used to verify the effect of the deletion in intron 6 on splicing were constructed using the N1-pEGFP vector (Clontech). The rat exon and intron fragments were obtained by PCR using the CH230-103H12 Bac clone (BacPac Resources) as a template and the primers shown in Table 1. All constructs were confirmed by sequencing at the University of Texas MD Anderson Cancer Center Core Facility or Macrogen (Seoul, Republic of Korea).

EMSA—This assay was performed as previously described (15) with minor modifications. Truncated human recombinant SOX9 (amino acid residues 1–300) and FLAG-tagged-SOX5

protein were used as binding proteins. The FLAG-tagged human SOX5 cDNA was expressed in 293T cells and purified by means of an anti-FLAG M2 affinity gel (A2220, Sigma) and FLAG peptide (F3290, Sigma) according to the manufacturer's protocol. 0.5 pmol of the 5′ Cy3-labeled 30-bp fragment in intron 6 or 29-bp fragment of the SOX9-binding site in intron 1 (Macrogen) was used as a probe (Table 1). The probe of a larger size (325-bp) was prepared by PCR using 5′ Cy3-labeled primers (Table 1) and intron 6 DNA as a template. In total, 2 μg of purified SOX9, 5 μg of FLAG-SOX5, 14 μg of nuclear extract from RCS cells, or 8 μg of nuclear extract from mouse rib chondrocytes were used as binding proteins. For competition assay by the non-labeled probe, 20× the amount of non-labeled oligos to the labeled probe was applied after the binding reaction. In some reactions, 2 μg of an anti-Sox9 antibody (Cell Signaling Technology #82630) or an anti-FLAG M2 antibody (F3165, Sigma) was added to the reaction. After the electrophoretic run was finished, the Cy3-labeled DNA was detected using a KETA M Series Imaging System (Wealtec Corp.). All experiments were performed at least three times, yielding very similar results. The experiment using mice was carried out in strict accordance with the recommendations in the Guide for the Care and Use of the Konkuk University Animal Care and Experimentation Community. All experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the Konkuk University (IACUC approval number KU11035).

ChIP-Seq and the ChIP-qPCR Assay—The ChIP-Seq experiments and analysis were performed as described elsewhere (7, 8). To measure the mRNA expression level, total RNA was extracted from RCS cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was prepared from the total RNA using a QuantiTect Reverse Transcription kit (Qiagen). qPCR was conducted using primers specific for each cDNA, with a SYBR Master Mix, SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK), on a ViiA7 Real-Time PCR system (Applied Biosystems) as previously described (9). The difference in C_t values (ΔC_t) between the C_t value of each sample and that of *GAPDH* was calculated. The ΔC_t value of each gene was then compared with that of each sample by the $\Delta\Delta C_t$ method. The primers are shown in Table 1. ChIP-qPCR was performed as previously shown (9), except with a Genomic DNA Extraction Kit (GeNet Bio, Chungcheongnam-do, Republic of Korea) instead of phenol/chloroform extraction to purify the ChIP DNA. The primers are also listed in Table 1; the probe location in intron 6 of the *Col2a1* gene is shown in Fig. 8*B*.

Knock-out Cells—To prepare deletion mutants of intron 6 or exon 5 of the *Col2a1* gene from RCS cells, the CRISPR/Cas9 system, by means of pSpCas (BB)-2A-puro (PX459) v2.0, which was a gift from Dr. Feng Zhang (Addgene plasmid #62988), was used (33). The CRISPR/Cas9 target site was selected using the target design software available in Dr. Zhang's laboratory. Mutant selection was performed according to the method previously reported using a Puromycin-SSA plasmid system (34). The INDEL of the *Col2a1* gene in puromycin-resistant colonies was detected by dideoxy sequencing (Macrogen) of each plasmid cloned by a T-easy Cloning Kit (Promega) after PCR amplification using the primers listed in Table 1.

Western Blotting—The total cell lysates dissolved in Laemmli sample buffer were applied to an SDS-7.5% polyacrylamide gel. The separated proteins were transferred to a PVDF membrane (Bio-Rad) and reacted with anti-SOX9 (ab185230, Abcam, Cambridge, UK), anti-β-actin (ab8227, Abcam), or anti-collagen II (ab34712, Abcam) followed by the reaction with HRP-labeled goat anti-rabbit IgG antibody (Enzo Life Sciences, NY). HRP was detected using an ECL detection kit (Thermo Fisher Scientific) and X-ray film.

Statistical Analysis—The values are expressed as the mean ± S.E. For the statistical analysis of the differences of independent samples ($n = 3$), Student's t tests were performed as appropriate. Significance was accepted at the 0.05 level of probability ($p < 0.05$). The p values are shown in each figure legend.

Author Contributions—H. Y., C-d. O., D. C., B. d. C., and J.-H. K. conceived and designed the experiments. H. Y. and C.-D. O. performed the experiments. H. Y., C.-d. O., and J.-H. K. wrote the paper.

Acknowledgment—We thank to Lizzie Hess, Diplomate Editor in the Life Sciences (ELS (D)), Scientific Editor, University of Texas MD Anderson Cancer Center for editing this manuscript.

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