Journal of Cellular Biochemistry Supplement 36:71–78 (2001)

Transcriptional Suppression of *Sox9* Expression in Chondrocytes by Retinoic Acid

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Abstract *SOX9* is a transcription factor that is expressed in chondrocytes and regulates expression of chondrocyte phenotype related genes. Expression of these genes is known to be suppressed by retinoic acid (RA). We, therefore, examined whether the *Sox9* gene expression is regulated by RA in chondrocytes. RA treatment suppressed *Sox9* mRNA expression in primary chondrocytes prepared from newborn mouse rib cartilage within 12 h and this suppression lasted at least up to 24 h. The RA suppression of *Sox9* mRNA levels was dose-dependent starting at 0.5 μM with a maximum at 1 μM. Nuclear run-on assays revealed that RA reduced the rate of transcription of *Sox9* gene. Finally, Western blot analysis indicated that RA suppressed SOX9 protein levels in these chondrocytes. Furthermore, overexpression of SOX9 reversed RA suppression of *Col2a1* enhancer activity. These observations indicate that RA suppresses *Sox9* gene expression in chondrocytes at least in part through transcriptional events. J. Cell. Biochem. Suppl. 36:71–78, 2001.

Key words: *SOX9*; chondrocyte; retinoic acid; transcription; type II collagen

INTRODUCTION

Sox9 is a member of the family of *Sox* (*Sry*-type HMG box) genes [Wright et al., 1993] which were first identified on the basis of a region with a high homology to *Sry* (Sex-determining region Y) [Gubbay et al., 1990]. This region encodes a 79 amino acid motif known as HMG box, which is responsible for sequence-specific DNA binding [Giese et al., 1992; Harley et al., 1994]. Several HMG box proteins are known to act as transcription factors [Jantzen et al., 1990] and some of the *Sox* genes have been shown to be expressed in a tissue specific manner during development [Wegner, 1999]. SOX9 is expressed predominantly in mesenchymal condensations throughout the embryo in the regions where deposition of cartilage matrix is taking place, suggesting its role in skeletal formation [Wright et al., 1995; Ng et al., 1997; Zhao et al., 1997]. In addition, mutations in human *SOX9* are observed in patients with campomelic dysplasia, characterized by skeletal malformation and XY sex reversal [Foster et al., 1994; Wagner et al., 1994; Schafer et al., 1995]. In mouse chimaeras, SOX9-/- cells are excluded from all the cartilaginous tissues during embryonic development and are present

Grant sponsor: Japanese Ministry of Education; Grant numbers: 11877357, 10044246, 10877223, 0930734; Grant sponsor: CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST); Grant sponsor: "Research for the Future" Program of the Japan Society for the Promotion of Science(JSPS); Grant numbers: 96100205; Grant sponsor: Traffic Medicine Foundation, Interdisciplinary Cancer Research Foundation, Inamori Foundation and NASDA, to MN.

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Received 4 August 2000; Accepted 2 November 2000

This article published online in Wiley InterScience, March 15, 2001.

in juxtaposed mesenchyme that does not express chondrocyte-specific markers such as procollagen type II, IX, XI, and aggrecan [Bi et al., 1999]. These observations indicate that SOX9 plays a key role in chondrogenesis and skeletogenesis.

Differentiation of chondrocytes is regulated by cytokine, hormone, cell adhesion, and biomechanical forces. Among these, RA has been reported to suppress expression of genes encoding chondrocyte-phenotype related proteins such as aggrecan and type II collagen (*Col2a1*) [Benya and Padilla, 1986; Horton et al., 1987b]. RA also affects other features of chondrocytes in culture, including the alteration of cell morphology [Brown and Benya, 1988] possibly related to its induction of the synthesis of a large amount of fibronectin [Bernard et al., 1984].

RA is a vitamin A metabolite, which not only modulates differentiation of chondrocytes but also regulates skeletal morphogenesis during embryonic development [Benya and Padilla, 1986; Horton et al., 1987b]. The RA activities are mediated by RARs and RXRs, which belong to the nuclear receptor superfamily [Mangelsdorf et al., 1995]. RARs and RXRs are encoded by at least three distinct genes, RAR α, -β, -γ, and RXR α, -β, -γ respectively [Mangelsdorf et al., 1992]. Mice lacking RARγ alone [Lohnes et al., 1993] or both RARα and RARγ [Lohnes et al., 1994] exhibit growth retardation, malformation, and homeotic transformations in their skeletons. Expression of a dominant-negative RAR in chondrogenic cells using *Col2a1* promoter causes retardation in skeletal formation and selective anterior transformation in their cervical vertebrae in mice [Yamaguchi et al., 1998]. Recently, it was reported that inactivation of the retinaldehyde dehydrogenase-2 gene (Raldh2), which encodes the earliest RA synthesis enzyme in the mouse embryos, results in shortening of the body in anterioposterior axis and loss of limb bud formation [Niederreither et al., 1999]. These observations indicate that RA is a potent modulator of chondrogenesis during skeletal morphogenesis.

SOX9 protein has been reported to bind specifically to the regulatory sequences located in the first intron of *Col2a1* gene to activate its expression in a cartilage specific manner in vitro [Lefebvre et al., 1997] and in vivo [Bell et al., 1997]. Based on these observations, we hypothesized that RA may regulate *Sox9* gene expression. The purpose of this study was to examine directly whether RA is able to influence *Sox9* gene expression in chondrocytes.

MATERIALS AND METHODS

Cell Culture

Chondrocytes were prepared from rib cartilage of 0 to 7 d-old ICR mice [Lefebvre et al., 1994]. Rib cages were incubated in 3 mg/ml collagenase (Sigma, St. Louis) in DMEM (GIBCO BRL, NY) for 30 min at 37°C, rinsed with PBS and then incubated in 3 mg/ml collagenase in DMEM at 37°C in a CO_2 incubator for 5 h. Undigested bony parts were discarded and primary schondrocytes were plated at 10^5 cells/cm^2 in tissue culture plastic dishes and were used within several days. These cells were cultured in DMEM supplemented with antibiotics (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) and 10% FBS (GIBCO BRL, NY). All-trans RA (Sigma, St. Louis) was dissolved in 95% ethanol. Control cultures received an equivalent amount of 95% ethanol.

Northern Analyses

An EcoRI-Bgl II 1.6kb fragment of the mouse *Sox9* cDNA was used as a probe [Wright et al., 1995]. An *Eco*RI 1.2 kb fragment of the glyceraldehyde-phosphate dehydrogenase (GAPDH) probe was used as a control. Total RNA was prepared according to the acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Aliquots of 10 μg of the total RNA per lane were electrophoresed in 1.0% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech, England) by electroblotting. Filters were prehybridized for 1 h at 42°C. Each cDNA was labeled using BcaBEST random primer labeling kit (Takara Shuzo Co., Ltd.,Tokyo) and [α-^{32}P] dCTP (NEN Life Science Products, Boston, MA). Hybridization was performed at 42°C for 24 h. Filters were washed in 2x SSC, 0.5% SDS for 20 min at room temperature and in 0.2x SSC, 0.5% SDS for 20 min at 65°C. Filters were exposed to X-ray film using intensifying screens at −80°C. The bands in the northern blot autoradiographs were quantified by desitometry and each value was normalized against that of the GAPDH band in the corresponding lane. The normalized

values obtained were used to evaluate the levels of mRNAs.

Nuclear Run-on Assay

Nuclei were isolated from control and 1μM RA-treated chondrocytes after 48 h in culture as previously reported [Horton et al., 1987b] [Derman et al., 1981]. The cells were rinsed with phosphate-buffered saline and harvested in cold phosphate-buffered saline. The cells were lysed in 2 ml RSB solution (10 mM KCl, 10 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$) by 10 strokes in a Dounce tissue homogenizer. The homogenates were allowed to swell for 10 min on ice. After adding 2 ml RSB + 0.6M sucrose, the cells were further subjected to lysis by 10 strokes in a homogenizer. The nuclei were spun down by centrifugation at 1500 rpm for 3 min at 4°C and were washed in 1 ml RSV+0.3M sucrose. Furthermore, the nuclei were spun down by centrifugation at 500 rpm for 1 min at 4°C and resuspended in 75μl Mg free RSB solution (10 mM KCl, 10 mM Tris-HCl PH 7.5).

For transcription, nuclei were incubated for 20 min at 25°C in a 100 μl 2x transcription mixture (100 mM Tris-HCl PH 7.5, 200 mM $(NH_4)_2SO_4$, 3.6 mM DTT, 3.6 mM $MnCl_2$), 2 μl of 33mM ATP, CTP, and GTP, 2 μl of 3 mM UTP, 5 μl α-[^{32}P]UTP(3000Ci/mmol, Amersham), and 80 units RNase inhibitor (RNasin, PROMEGA, Madison). The reaction was then treated with RNAase-free DNAase (Boehringer Mannheim, Germany) in the presence of 100 μg yeast transfer RNA (Boehringer Mannheim, Germany) for 15 min at 25°C, after which the sample was deproteinized with 800 μg proteinase K (Sigma, St. Louis, MO) in the presence of 1% SDS for 60 min at 4°C. Phenol/chloroform extraction was performed and 0.1 volume of 3M NaCl was added to the aqueous phase and precipitated with 2 volumes of ethanol on dry ice-ethanol bath. After centrifuge at 15,000 rpm for 5 min, the pellet was washed with 80% ethanol at −20°C and resuspended in 6M GuHCl and was allowed to hydrate for 15 min. The sample was mixed with 250 μl ethanol and was allowed to precipitate at −20°C overnight. After centrifuge, the pellet was rinsed with 80% ethanol at −20°C and resuspended in H_2O.

Linearized plasmid DNA (10 μg) was dissolved in 170 μl Tris-HCl (100 mM, pH 7.4), 30 μl 2N NaOH, and 100 μl 20x SSC as previously reported [Derman et al., 1981; Horton et al., 1987b]. The DNA was denatured at 80°C for 10 min, then cooled and neutralized with 40 μl of 2M Tris-HCl, pH 7.0. The DNA was blotted onto nylon filter (GeneScreen, NEN Life Science, Boston, MA) using a filtration manifold apparatus (Schleicher & Schuell). The filters were blotted, dried overnight, and prehybridized in 50% formamide, 5x SSC, 5X Denhaldt's solution, 10 mM EDTA, 0.1% SDS, 50 mM Tris-HCl pH 7.5, 20 μg/ml yeast transfer RNA, 10 μg/ml poly A and 100 μg/ml salmon sperm DNA at 42°C overnight. The filters were hybridized in a fresh solution containing the radiolabeled RNA isolated from control and RA-treated nuclei at 42°C for 3 days. Then, the filters were washed in 2X SSC and 0.1% SDS for 10 min at room temperature, followed by wash in 0.2x SSC and 0.1% SDS for 10 min at 60°C. The filters were dried and then exposed to X-ray films.

Western Blotting

Chondrocytes were cultured for 24 h in the presence or absence of RA and then scraped in a lysis buffer (150 mM NaCl, 1 mM PMSF, 1% Triton X100, 1 x protease inhibitor Complete (Boehringer Mannheim, Germany), 20mM Tris pH 8.0). The supernatants were used for Western blotting. Extracts were fractionated on 10% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Molsheim, France). The filters were blocked in 5% skim milk in Tris-buffered saline overnight and incubated with a 1/5000 dilution of anti-SOX9 antibody and then incubated with a 1/4000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody. The SOX9 protein was detected by using ECL kit (Amersham Pharmacia Biotech, England). Anti-SOX9 antibody was produced in a rabbit which was injected with a chemically synthesized peptide coupled to KLH (keyhole limpet haemocyanin). This peptide corresponds to the amino acid sequence 486 to 509 at the C-terminus of human SOX9 protein. HA-tagged full length SOX9 protein was used as a positive control and luciferase protein was used as a negative control. For the production of full-length human SOX9, PBS-HA-SOX9 plasmid was constructed by PCR amplification using SOX9 cDNA as a template [Mertin et al., 1999]. SOX9 protein (509 amino acids) was produced using the TNT T3-coupled rabbit reticulocyte lysate system (Promega, Madison, WI).

CAT Assay

Chondrocytes were plated in 6-well cluster plates (35 mm in diameter) at 60–70% confluence. Twelve hours later, the cells were cotransfected with 1 μg of pCII4-C reporter plasmid containing promoter and enhancer regions of *Col2a1* gene [Horton et al., 1987a] and a SOX9 expression vector containing 1.9kb *Sox9* cDNA [Wright et al., 1995] or pSG5 vector plasmid. The cells were then cultured in the presence or absence of 1 μM RA and were harvested 48 h after the initiation of RA treatment. Transfection was performed using FuGENE6 (Boehringer Mannheim, Germany). pSV2-CAT plasmid was used as a positive control. Cell extracts were then prepared and used for CAT assay. Protein concentrations in the lysates were determined by the Coomassie Brilliant Blue G method. Equivalent amounts of the cellular proteins were incubated in a reaction buffer (0.25M Tris-HCl, 40mM acetyl CoA (Sigma), [^{14}C] chloramphenicol (Amersham Corp. Tokyo)) for 2 h at 37°C. The levels of acetylation were examined by TLC followed by autoradiography. Quantitation of the acetylation levels was performed by using a Bioimaging Analyzer BAS 2000 system (Fuji Film Inc., Tokyo, Japan). Transfection efficiency was monitored in a part of experiments by cotransfection with PGL2-Control LUC (Promega).

RESULTS

RA Suppresses Sox9 mRNA Expression in Chondrocytes

Sox9 mRNA is expressed at high levels in the primary cultures of chondrocytes, as previously reported [Lefebvre et al., 1997]. *Sox9* mRNA was detected as a 4.7 kb band in these cells (Fig. 1). Treatment with RA suppressed *Sox9* mRNA levels in a dose-dependent manner: suppression of the *Sox9* mRNA expression was first observed at 0.5 μM RA and was maximal at 1 μM after 24 h treatment (Fig. 1). RA did not affect GAPDH mRNA levels, indicating specificity of its effect on *Sox9* mRNA. The RA effect was time-dependent with *Sox9* mRNA abundance decreasing by about 80% within 12h remaining low at least up to 24 h (Fig. 2).

RA suppresses Sox9 expression at the transcriptional level. Since RA binds to a nuclear receptor which acts as a transcription factor, we examined whether RA treatment affects *Sox9* gene transcription. Nuclear run-on assays were performed using primary chondrocytes. The rate of *Sox9* gene transcription was reduced by about 80% in the nuclei isolated from RA-treated chondrocytes compared to that in control cell nuclei (Fig. 3). The rate of GAPDH gene transcription was similar regardless of the presence or absence of RA (Fig. 3), indicating the specificity of RA effects on the rate of *Sox9* gene transcription. No signal was detected in the pSG5 spots used as negative control. These results indicate that RA suppresses *Sox9* gene expression at least in part at transcriptional level.

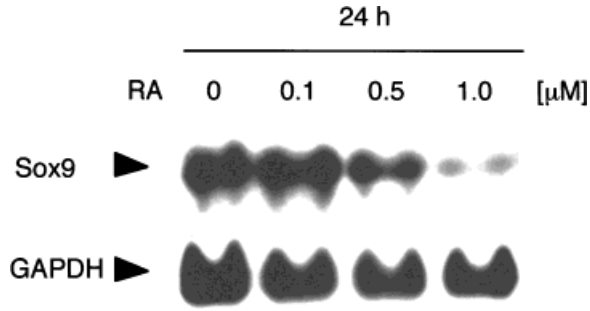

Fig. 1. Dose-dependence of the effects of RA on *Sox9* mRNA levels in chondrocytes. Primary cultures of chondrocytes were treated with the indicated doses of RA for 24 h. *Sox9* mRNA expression was examined by Northern blot analysis as described in Materials and Methods. The data represent one of three experiments with similar results.

RA does not affect *Sox9* mRNA stability. To examine whether RA also affects *Sox9* mRNA stability, a transcription inhibitor, actinomycin D was added to culture medium 15 min before the addition of vehicle or 1 μM RA. The half life of the *Sox9* mRNA in the absence of RA was about 2.5 h and this was similar in the

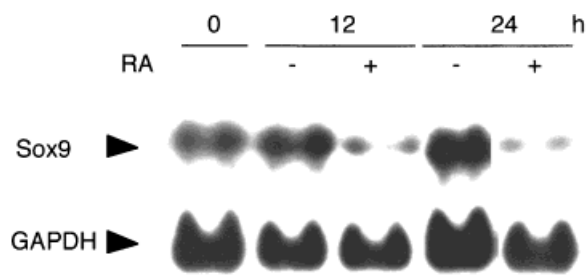

Fig. 2. Time-course of the effects of RA on *Sox9* mRNA levels in chondrocytes. Primary cultures of chondrocytes were treated with 1 μM RA for the indicated periods of time. *Sox9* mRNA expression was examined by Northern blot analysis as described in Materials and Methods. The data represent one of three experiments with similar results.

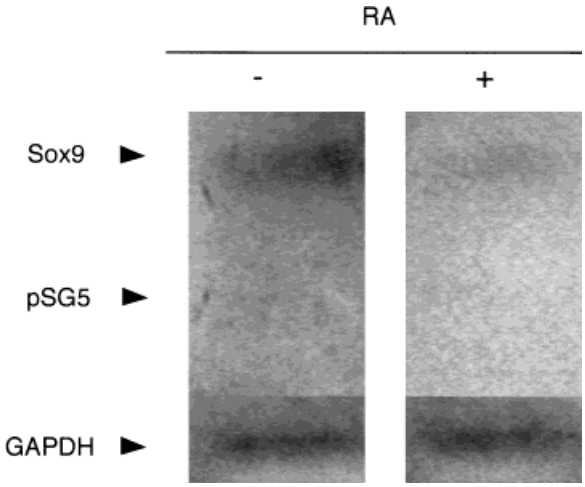

Fig. 3. Nuclear run-on assay indicates that RA suppresses transcription of *Sox9* Nuclear run-on assays were performed using control chondrocyte nuclei and nuclei from chondrocytes treated for 48 h with 1 μM RA as described in Materials and Methods. RA treatment decreased the levels of *Sox9* transcripts. The level of GAPDH transcript was not affected by RA. As negative control, pSG5 vector plasmid was used.

presence of RA (Fig. 4), indicating that RA does not affect Sox9 mRNA stability.

RA decreases SOX9 protein expression. We next determined whether the effect of RA on *Sox9* mRNA levels is reflected in protein expression levels, using Western blot analysis. Treatment with RA decreased the abundance of SOX9 protein, which migrated

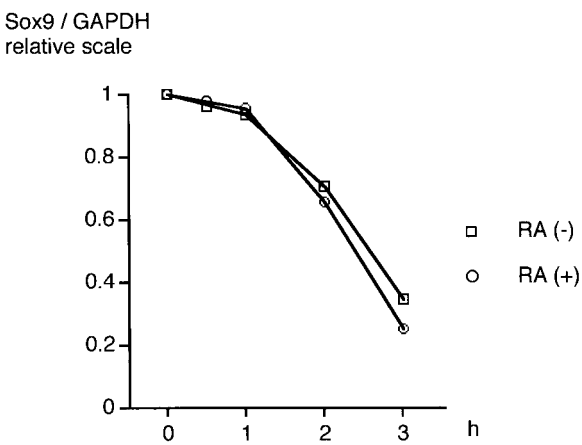

Fig. 4. *Sox9* mRNA stability in the presence of RA. Primary cultures of chondrocytes were treated with 0.2 μg/ml actinomycin D (AD) 15 min prior to the addition of vehicle (−) or 1 μM RA (+). Total RNA was extracted at 0, 0.5. 1, 2, and 3 h after the addition of vehicle or RA and was subjected to Northern blot analysis as described in Materials and Methods. The ratios of *Sox9/ GAPDH* are indicated. The figure represents one of two experiments with similar results.

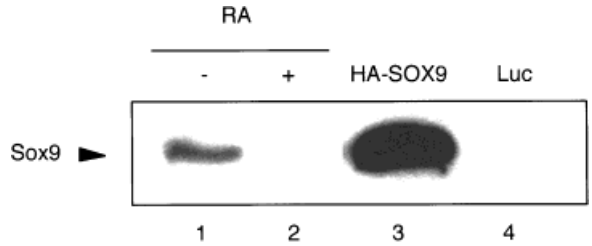

Fig. 5. Suppression of SOX9 protein levels by RA. Primary cultures of chondrocytes were treated with 1 μM RA for 24 h. Cell extract was prepared and was subjected to Western blot analysis as described in Materials and Methods. HA-tagged full length SOX9 protein which migrated as a 69 kDa band was used as a positive control and luciferase protein was used as a negative control. The data represent one of three experiments with similar results.

as a 69 kDa band (Fig. 5). These experiments indicate that RA suppression of *Sox9* gene transcription rate leads not only to the reduction of its mRNA levels but also to the down-regulation of the SOX9 protein levels.

***SOX9* overexpression blocks RA suppression of *Col2a1* enhancer activity.** RA suppresses *Col2a1* enhancer activity in chondrocytes [Horton et al., 1987a]. We therefore examined whether SOX9 mediates this suppression using transient transfection assays. As shown in Figure 6, RA suppressed the activity of pCII4-C CAT reporter construct, which contains chondrocyte-specific Sry-Sox consensus sequence within the enhancer region of *Col2a1* gene as previously reported [Horton et al., 1987a]. SOX9 overexpression blocked RA suppression of the type II procollagen enhancer activity (Fig. 6). Neither RA treatment nor SOX9 overexpression altered pSV2-CAT activity, indicating the specificity of the effects of RA and SOX9 on the *Col2a1* enhancer (data not shown).

DISCUSSION

We show here that expression of SOX9, a transcription factor essential for chondrocyte-specific phenotype expression, is suppressed by RA in chondrocytes. We also found that RA regulates *Sox9* gene expression at least in part via transcriptional events.

SOX9 inhibition requires at least 500 nM whereas the physiological plasma levels of retinoic acid are about 3 nM. Thus, it appears that the observations presented in this paper may not represent a physiological but rather a pharmacological effect. Certainly the RA concentrations required to modulate Sox9 activity

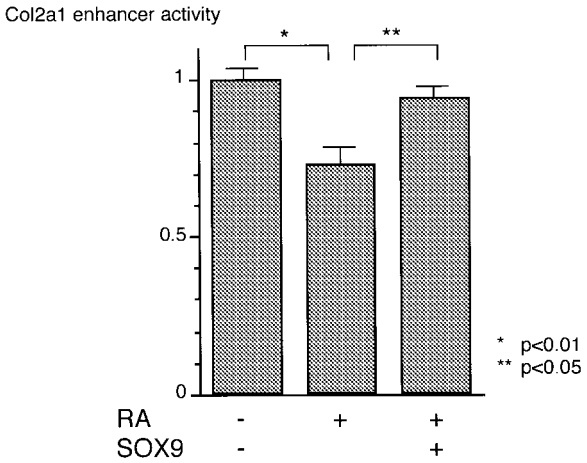

Fig. 6. *Sox9* overexpression blocks RA suppression of *Col2a1* enhancer activity. Primary cultures of chondrocytes were co-transfected with 1 μg of *Col2a1*-promoter/enhancer-CAT reporter plasmid and 0.5 μg of SOX9 expression vector or 0.5 μg of pSG5 vector plasmid. Then the cells were cultured in the presence or absence of 1 μM RA for 48 h. CAT activity was determined as described in Materials and Methods. Experiments were repeated twice in triplicate with independent preparations of cell extracts. The representative data was shown. Data are presented as mean ± S.D. (n=3). *, $P < 0.01$, **, $P < 0.05$, by unpaired Student's *t*-test.

are comparable with those previously reported to affect chondrogenesis in vivo and in vitro.

Although RA induces dedifferentiation of chondrocytes biochemically and morphologically by downregulating expression of genes specifically expressed in these cells, there have been only a limited number of reports on the molecular mechanism(s) of this RA suppression of chondrocyte dedifferentiation. RA was reported to suppress the expression of cartilage-derived RA-sensitive protein (CD-RAP), a molecule expressed exclusively in chondrocytes and co-downregulated with type II collagen by RA. This RA suppression of CD-RAP gene is mediated by RA enhancement of AP-2 [Xie et al., 1998], which plays a crucial role in the control of gene expression in response to a variety of cell diffferentiation signals [Mitchell et al., 1991]. The AP-2 null mice die perinatally with cranio-abdominoschisis and severe dysmorphogenesis of the face and skull [Schorle et al., 1996; Zhang et al., 1996]. However, AP-2 is a ubiquitous transcription factor and, therefore, no cell-specific transcriptional factors and/or cell-specific post-transcriptional events have been identified to be involved in the RA action in chondrocytes. Our data indicate that RA downregulates expression of SOX9, a chondrocyte-related transcription factor which directly regulates a suite of genes encoding major cartilage-specific extracellular matrix proteins such as type II collagen [Bell et al., 1997; Lefebvre et al., 1997].

SOX9 was also reported to bind to a SOX consensus sequence in the CD-RAP promoter and enhance its activity in chondrocytes [Xie et al., 1999]. Our present data suggest that SOX9 may mediate the effect of RA on CD-RAP expression.

RA suppressed the transcriptional activity of a *Col2a1*-CAT construct that contains *Sry*/*Sox*-consensus sequences from the first intron fragment of the *Col2a1* gene. However, the 30% decrease in *Col2*-CAT activity was not as great as the suppression of endogenous *Col2a1* mRNA expression (data not shown). As comparing the activity of a construct in transient expression experiments with the activity of the endogenous gene in its chromatin environment does not allow clear conclusions, the reason for the relatively small levels of the suppression in Col2-Cat activity still remains to be elucidated.

Although it was reported that most of the elements necessary for proper expression of *Sox9* in skeletal tissues are located up to 350 kb upstream of *Sox9* [Wunderle et al., 1998], and that RA downregulates transcription of several genes [Horton et al., 1987b], no consensus sequences have been yet identified as negative RA response elements (nRERs) except that RARE acts as a negative regulator of the keratin K5, K6, and K17 genes [Radoja et al., 1997]. Our data suggest the presence of nRERs within the *Sox9* gene though the exact nature of these elements is yet to be clarified.

We have previously reported that RA enhances SOX9 mRNA expression in the cartilaged-derived cell line, TC6 [Sekiya et al., 2000]. Responsiveness to RA may depend on chondrocyte subtypes. Primary chondrocytes consist of heterogeneous subpopulations whereas TC6 cells represent a homogeneous population of chondrocytes at a certain stage, in which RA may up-regulate SOX9. Further analysis on the RA effects on chondrocytes in vivo and /or in vitro at different time course and doses would be required to reconcile these observations.

In conclusion, we show here that SOX9 is one of the targets of RA regulation of chondrocyte function. Since SOX9 is the key transcription factor for the regulation of *Col2a1* gene expression, the pathway identified in this report would

at least partially mediate the activity of RA in controlling chondrocyte differentiation.

REFERENCES

Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS. 1997. SOX9 directly regulates the type-II collagen gene. Nat Genet 16:174–178.

Benya PD, Padilla SR. 1986. Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen: the modulated phenotype differs from that produced by subculture. Dev Biol 118:296–305.

Bernard BA, De Luca LM, Hassell JR, Yamada KM, Olden K. 1984. Retinoic acid alters the proportion of high mannose to complex type oligosaccharides on fibronectin secreted by cultured chondrocytes. J Biol Chem 259:5310–5315.

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. 1999. Sox9 is required for cartilage formation. Nat Genet 22:85–89.

Brown PD, Benya PD. 1988. Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced reexpression. J Cell Biol 106:171–179.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Derman E, Krauter K, Walling L, Weinberger C, Ray M, Darnell JE, Jr. 1981. Transcriptional control in the production of liver-specific mRNAs. Cell 23:731–739.

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372:525–530.

Giese K, Cox J, Grosschedl R. 1992. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell 69:185–195.

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346:245–250.

Harley VR, Lovell-Badge R, Goodfellow PN. 1994. Definition of a consensus DNA binding site for SRY. Nucleic Acids Res 22:1500–1501.

Horton W, Miyashita T, Kohno K, Hassell JR, Yamada Y. 1987a. Identification of a phenotype-specific enhancer in the first intron of the rat collagen II gene. Proc Natl Acad Sci USA 84:8864–8868.

Horton WE, Yamada Y, Hassell JR. 1987b. Retinoic acid rapidly reduces cartilage matrix synthesis by altering gene transcription in chondrocytes. Dev Biol 123:508–516.

Jantzen HM, Admon A, Bell SP, Tjian R. 1990. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature 344:830–836.

Lefebvre V, Garofalo S, Zhou G, Metsaranta M, Vuorio E, De Crombrugghe B. 1994. Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. Matrix Biol 14:329–335.

Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. 1997. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 17:2336–2346.

Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. 1993. Function of retinoic acid receptor gamma in the mouse. Cell 73:643–658.

Lohnes D, Mark M, Mendelsohn C, Dolle P, Dierich A, Gorry P, Gansmuller A, Chambon P. 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. Development 120:2723–2748.

Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev 6:329–344.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al. 1995. The nuclear receptor superfamily: the second decade. Cell 83:835–839.

Mertin S, McDowall SG, Harley VR. 1999. The DNA-binding specificity of SOX9 and other SOX proteins. Nucleic Acids Res 27:1359–1364.

Mitchell PJ, Timmons PM, Hebert JM, Rigby PW, Tjian R. 1991. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes Dev 5:105–119.

Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, Bell DM, Tam PP, Cheah KS, Koopman P. 1997. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 183:108–121.

Niederreither K, Subbarayan V, Dolle P, Chambon P. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 21:444–448.

Radoja N, Diaz DV, Minars TJ, Freedberg IM, Blumenberg M, Tomic-Canic M. 1997. Specific organization of the negative response elements for retinoic acid and thyroid hormone receptors in keratin gene family. J Invest Dermatol 109:566–572.

Schafer AJ, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, et al. 1995. The role of SOX9 in autosomal sex reversal and campomelic dysplasia. Philos Trans Royal Soc London-Series B: Biol Sci 350:271–277, Discussion 277-278.

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature 381:235–238.

Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, Nifuji A, Noda M. 2000. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem 275:10738–10744.

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79:1111–1120.

Wegner M. 1999. From head to toes: the multiple facets of Sox proteins. Nucleic Acids Res 27:1409–1420.

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P.

1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 9: 15–20.

Wright EM, Snopek B, Koopman P. 1993. Seven new members of the Sox gene family expressed during mouse development. Nucleic Acids Res 21:744.

Wunderle VM, Critcher R, Hastie N, Goodfellow PN, Schedl A. 1998. Deletion of long-range regulatory elements upstream of SOX9 causes campomelic dysplasia. Proc Natl Acad Sci USA 95:10649–10654.

Xie WF, Kondo S, Sandell LJ. 1998. Regulation of the mouse cartilage-derived retinoic acid-sensitive protein gene by the transcription factor AP-2. J Biol Chem 273:5026–5032.

Xie WF, Zhang X, Sakano S, Lefebvre V, Sandell LJ. 1999. Trans-activation of the mouse cartilage-derived retinoic acid-sensitive protein gene by Sox9. J Bone Min Res 14:757–763.

Yamaguchi M, Nakamoto M, Honda H, Nakagawa T, Fujita H, Nakamura T, Hirai H, Narumiya S, Kakizuka A. 1998. Retardation of skeletal development and cervical abnormalities in transgenic mice expressing a dominant-negative retinoic acid receptor in chondrogenic cells. Proc Natl Acad Sci USA 95:7491–7496.

Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature 381:238–241.

Zhao Q, Eberspaecher H, Lefebvre V, De Crombrugghe B. 1997. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. Dev Biol 209:377–386.