Sox9 and p300 Cooperatively Regulate Chromatin-mediated Transcription*§

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Takayuki Furumatsu†, Masanao Tsuda‡, Kenji Yoshida‡, Noboru Taniguchi‡, Tatsuo Ito‡, Megumi Hashimoto‡, Takashi Ito‡, and Hiroshi Asahara§§

From the ¶Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037, the §Department of Biochemistry, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan, the ‡National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan, and the †Solution Oriented Research for Science and Technology (SORST) Project, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Chromatin structure is a fundamental component of gene regulation, expression, and cellular differentiation. We have previously reported that the multifunctional coactivator p300 is a member of the Sox9 (Sry-type high mobility group box 9)-related transcriptional apparatus and activates Sox9-dependent transcription during chondrogenesis. However, the mechanism of synergy between Sox9 and p300 in chromatin-mediated transcription has not been elucidated. In the present study we investigated the activity of Sox9 and p300 on chromatinized templates in vitro. Recombinant Sox9 was shown to be associated with several transcriptional cofactors including p300. In vitro transcription assays revealed that p300 potentiated Sox9-dependent transcription on chromatinized DNA and, importantly, was associated with hyperacetylated histones. Consistent with these results, the histone deacetylase inhibitor trichostatin A stimulated the expression of Sox9-regulated cartilage matrix genes and induced histone acetylation around the enhancer region of the collagen α1 (II) gene in chondrocytes. These findings suggest that Sox9 interacts with chromatin and activates transcription via regulation of chromatin modification.

Chondrocyte differentiation from mesenchymal stem cells (MSCs) to hypertrophic chondrocytes is a dynamic process in endochondral bone formation. The sequential differentiation and maturation steps of chondrocytes are regulated by several transcription factors such as Sox5/6/9 and runt-related gene Runx2 (1–3). Sox9 (Sry-type high mobility group box 9) plays an essential role in establishing the condensation of prechondrogenic mesenchymal cells and initiating chondrocyte differentiation (1, 2). Mutations that inactivate SOX9 cause the skeletal malformation syndrome campomelic dysplasia (4, 5). Expression of the α1 chain of type II collagen (Col2α1), a major component of the cartilage extracellular matrix, is regulated by Sox9 through the Sox9-binding site on the Col2α1 enhancer region (6). However, Sox9-dependent transcriptional regulation in chondrogenesis seems to require other additional cofactors (7). We have previously demonstrated that p300/CREB-binding protein (CBP), peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α), and Smad3 associate with Sox9 and enhance the transcriptional activity of Sox9 during chondrogenesis (8–10). However, little is known about the mechanism of Sox9-driven activation.

The eukaryotic genome is packaged into chromatin in which DNA wraps around the surface of a histone octamer (two sets of histone H2A, H2B, H3, and H4) and forms the nucleosome-repeated structure (11–13). The chromatin structure controls gene expression and other fundamental cellular processes (11–13) by inhibiting the access of regulatory factors to DNA. These inhibitory effects can be reversed by the activity of chromatin remodeling factors.

In vitro chromatin assembly and transcription assays have been very useful in elucidating the effects of chromatin structure on gene expression. Several proteins have been identified as important factors for regulating chromatin assembly and modification. In vitro chromatin assembly requires factors such as nucleosome assembly protein-1 (NAP-1), which acts as a histone-shuttling protein (14, 15). Additionally, the ATP-utilizing chromatin assembly and remodeling factor (ACF), consisting of Acf1 and Isw1 subunits, assembles periodic nucleosome arrays on histone-attached DNA in an ATP-dependent process (15, 16).

Factors that regulate histone modifications (including acetylation, methylation, phosphorylation, and ubiquitylation) have a critical role in regulating chromatin structure and, by extension, transcription (11–13, 17–19). In some cases DNA-bound transcription factors exert higher transcriptional potentials on acetylated chromatin (20, 21). In transcriptional events regulated by CREB or MyoD, for example, the acetylation of histone tails is a crucial step for relaxing the condensed chromatin structure leading to activation. CBP/p300 has intrinsic histone acetyltransferase (HAT) activity and modulates the function of its associated transcription factors (22–24). In addition to its interaction with transcription factors, CBP/p300 also plays a central role in cellular differentiation (21, 22, 25–28).

In a previous study we focused on p300 and its closely related analog, CBP, to investigate the involvement of these molecules in chondrogenesis (8). However, the precise mechanism by which CBP/p300 facilitates the transactivation process during chondrogenesis is not clear. In this study we demonstrate that Sox9 regulates transcription of cartilaginized DNA through the acetylation of histones by p300.
Sox9-dependent Regulation of Chromatin

MATERIALS AND METHODS

Cells, Plasmids, and Antibodies—Human chondrocyte stem cells (Cambrex) were maintained as described previously (10). Human chondrocytes were isolated and cultured as described earlier (29). MSCs (passages 3–6) and chondrocytes (passages 2–3) were used for these experiments. A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line (10). Plasmids encoding rat Sox9 (1–507) (full-length), 1–327, and 182–507 (numbers correspond to amino acid distributions) were used (8). p300 and the HAT-deficient mutant of p300 (p300DHT) were gifts from Tso-Pang Yao (30). The Col2a1 reporter plasmid (4X 48-p89luc), which contains four copies of the 48-bp Col2a1 enhancer element, and FLAG-tagged Smad3/4 were generous gifts from Benoît de Crombrugghe and Takeshi Imamura, respectively (31, 32). 12X 48-pGL3P was constructed using three sets of BamHI and BglII-cleaved 4X 48 fragments subcloned into pGL3-Promoter (Promega). PCR fragments of FLAG-tagged Sox9 (8) and FLAG-tagged Smad3/4 were subcloned into the BamHI and XhoI sites of the vector pENTR3C (Invitrogen). The antibodies used were anti-FLAG M2 (Sigma), glyceraldehyde-3-phosphate dehydrogenase (Ambion), Smad2/3 (Upstate), Smad4 (Cell Signaling), and Sox9 (Chemicon).

RNA Isolation and PCR—Total cellular RNA was extracted using RNeasy mini kits (Qiagen). RNA samples (500 ng) were reverse-transcribed to cDNA as described previously (33). The cDNAs were subjected to PCR amplification in the presence of COL2A1 or GAPDH primer pairs (10). Chondrocytes were treated with 300 nM histone deacetylase inhibitor trichostatin A (TSA; Sigma). Histidine-tagged p300 (p300DHAT) were gifts from Tso-Pang Yao (30). The vector pENTR3C was generated using Dual-Luciferase reporter assay system (Promega) as described previously (10). The assays were performed in triplicate.

Chromatin Assembly and Micrococcal Nuclease Assay—Chromatin assembly and micrococcal nuclease digestion analyses were performed as described (37) using 12X 48-pGL3P. For chromatin reconstitution, standard reactions (20 µl) containing plasmid (150 ng), histones (100 ng), NAP-1 (500 ng), ISWI/Act5 (0.65 ng each), ATP (3 mM), and ATP regeneration systems (30 mM phosphocreatine and 20 ng of creatine phosphokinase) were incubated at 30 °C for 4 h. In micrococcal nuclease assays, chromatinized plasmids (300 ng) were digested with micrococcal nuclease (0, 0.02, and 0.04 units/15 µl) for 5 min at 37 °C. 

In Vitro Transcription and S1 Nuclease Assay—After chromatin assembly, standard reactions (12X 48-pGL3P, 150 ng) were incubated with Sox9 (10 ng), p300 (30 ng), and acetyl-coenzyme A (Acyl-CoA, 5 µM) for 30 min at 30 °C. For in vitro transcription, SW1353 cells were transfected using FuGENE6 (Roche Applied Science). pRL-CMV (5 ng, Promega) was used as an internal control. The cells were harvested for 24 h after transfection, and then the luciferase activities were analyzed using Dual-Luciferase reporter assay system (Promega) as described previously (10). The assays were performed in triplicate.

Histone Acetyltransferase Assay—After histone acetyltransferase assay, chromatin assembly (12X 48-pGL3P, 600 ng), purified Sox9 (20 ng) and/or p300 (60 ng) was added to the reaction mixtures with [3H]-Acetyl-CoA (1 µM, PerkinElmer Life Sciences) and incubated for 1 h at 30 °C in the absence of unlabeled Acetyl-CoA. Reactions were stopped by boiling in loading buffer and analyzed on 15% SDS-polyacrylamide gels. The gels were stained with GelCode Blue reagents (Pierce) and then dried and exposed to autoradiography. Each experiment was performed at least three times.

Histone Deacetylase Assay—After histone deacetylase assay, chromatin assembly (12X 48-pGL3P, 600 ng), purified Sox9 (20 ng) and/or p300 (60 ng) was added to the reaction mixtures with [3H]-Acetyl-CoA (1 µM, PerkinElmer Life Sciences) and incubated for 1 h at 30 °C in the absence of unlabeled Acetyl-CoA. Reactions were stopped by boiling in loading buffer and analyzed on 15% SDS-polyacrylamide gels. The gels were stained with GelCode Blue reagents (Pierce) and then dried and exposed to autoradiography. Each experiment was performed at least three times.

Chromatin Immunoprecipitation Assay—Human chondrocytes (2X 10^5 cells) were maintained in the presence or absence of TSA (300 nM) for 4 h. The procedures used for analysis were described previously (29) except for protein A beads and anti-acetyl-histone H3 (Lys-9) and H4 (Lys-8) antibodies (Cell Signaling). Rabbit IgG was used as a control. Input fractions were loaded as 10% volume of samples. PCR analyses transforming growth factor-β (R&D Systems). Purified proteins were assessed by silver stain (Bio-Rad) and Western blotting analyses.

Electrophoretic Mobility Shift Assay—An electrophoretic mobility shift assay was performed essentially as described previously (31). The Col2a1 enhancer probe containing the Sox9-binding site (in capital letters) was generated by annealing the oligonucleotides 5’-gctgctgagaaaagcccCATTTCAgggac-3’ and 5’-gctctctCTGAGAATGggcttt-ctctaaggc-3’. Probes were 32P end-labeled using T4 polynucleotide kinase (Invitrogen). Purified Sox9 (30 ng) was incubated with the labeled probe (0.8 pmol). The unlabeled Col2a1 enhancer probe (16 pmol) was used as a competitor. In supershift analysis a 15-min treatment with anti-Sox9 antibody (0.2 µg) was performed before protein-DNA binding reactions.

Luciferase Reporter Assay—12X 48-pGL3P was used as a reporter gene for investigating the Sox9-dependent transcriptional activity. Plasmids (20 ng, except for 60 ng of p300) were transiently transfected into SW1353 cells using FuGENE6 (Roche Applied Science). pRL-CMV (5 ng, Promega) was used as an internal control. The cells were harvested for 24 h after transfection, and then the luciferase activities were analyzed using Dual-Luciferase reporter assay system (Promega) as described previously (10). The assays were performed in triplicate.

Histone acetylation and chromatin assembly were performed as described (37) using 12X 48-pGL3P. For chromatin reconstitution, standard reactions (20 µl) containing plasmid (150 ng), histones (100 ng), NAP-1 (500 ng), ISWI/Act5 (0.65 ng each), ATP (3 mM), and ATP regeneration systems (30 mM phosphocreatine and 20 ng of creatine phosphokinase) were incubated at 30 °C for 4 h. In micrococcal nuclease assays, chromatinized plasmids (300 ng) were digested with micrococcal nuclease (0, 0.02, and 0.04 units/15 µl) for 5 min at 37 °C.
were performed using the specific primer set for amplifying the COL2A1 enhancer region (29).

RESULTS

Recombinant Sox9 Associates with p300 and Binds to the Col2a1 Enhancer in Vitro—Col2a1 was detected in human chondrocytes but not in MSCs (Fig. 1, A and B). To assess the role of CBP/p300 in Sox9-dependent transcription during chondrogenesis, we first compared the amount of Sox9 complex in chondrocytes and human MSCs. Both Sox9 and p300 were highly expressed in mature chondrocytes but only weakly detected in MSCs (Fig. 1C). Higher amounts of Smad2/3/4 were also detected in the nuclear extracts of chondrocytes (supplemental Fig. 2, available in the on-line version of this article) and components from the SOX9 and p300, Smad2/3, and PGC-1α were also detected in the nuclear extracts of chondrocytes (supplemental Fig. 2, available in the on-line version of this article). The correlation between the high expression of p300 and Col2a1 in chondrocytes supports our previous findings that p300 is involved in Sox9-dependent transcription of its target gene, Col2a1 (8). Because p300 can acetylate histones and activate transcription, we monitored the effect of p300 on Sox9-dependent transcriptional initiation using a chromatinized target gene. To do this, we employed an in vitro reconstituted transcription system. First, we purified histones from HeLa cells (supplemental Fig. 2A, available in the on-line version of this article) and components from baculovirus, namely chromatin assembly-related molecules (NAP-1 and ACF complex; supplemental Fig. 2, B and C), Sox9 (supplemental Fig. 2D), and p300 (supplemental Fig. 2E). Purified NAP-1 and ACF assembled chromatin in the presence of DNA, histones, and ATP regenerating chromatins. We verified the assembly of chromatin in vitro by micrococcal nuclease digestion assay (data not shown).

We also examined whether purified Sox9 would interact with cofactors p300 and tested the ability of recombinant Sox9 to bind to the Col2a1 gene by an electrophoretic mobility shift assay (data not shown) as reported previously (8–10). Based on these results, we decided to analyze the transcriptional activity of the Sox9 complex on a chromatinized template.

Sox9 and p300 Cooperatively Activate the Chromatin-mediated Transcription in Vitro—We assessed the formation of the Sox9 protein complex on chromatin assembled in vitro using several different nuclear extracts. Consistent with our previous reports (8–10), endogenous p300, Smad2/3, and PGC-1α were co-immunoprecipitated with recombinant Sox9 in nuclear extracts from matured chondrocyte and SW1353 cells (Fig. 2). However, these proteins were not co-immunoprecipitated with purified Sox9 in MSC nuclear extracts (data not shown). These findings demonstrate that Sox9-associated molecules are present during chondrogenesis.

Next, we investigated the function of the Sox9 complex in vitro using chondrocyte and SW1353 extracts on chromatinized template DNA. In our assays with chromatinized templates, Sox9 was able to activate transcription of target DNA (Fig. 3B, lane 8). Consistent with the presence of Sox9 in this nuclear extract (Fig. 2), p300 was able to weakly activate transcription (Fig. 3B, lane 9), which may be due to complex formation between endogenous Sox9 and recombinant p300.

In fact, the addition of p300 potentiated the transcriptional activation by recombinant Sox9 (Fig. 3B, lane 10), clearly demonstrating that both p300 and Sox9 were necessary for activation of chromatin-mediated transcription (Fig. 3B, lane 10). In contrast, no significant differences were observed between Sox9- and Sox9/p300-treated samples in histone-free closed circular plasmids (Fig. 3B, section labeled Histone-free DNA). We confirmed these results in vivo using a reporter plasmid containing tandem copies of the Sox9 binding site. In reporter assays, the addition of p300 increased the relative luciferase activity in a Sox9-dependent manner, but not in assays using the p300-HAT-deficient mutant p300DHAT (Fig. 3C, line labeled Sox9, bars labeled 1–507). Sox9 182–507, which lacks the DNA-binding domain, was unaffected by the presence or absence of p300 (Fig. 3C, line labeled Sox9, bars labeled 182–507). In addition, the p300-binding domain of Sox9 (amino acids 328–507) was necessary for the p300-mediated transactivation (Fig. 3C, line labeled Sox9, bars labeled 1–327). These results suggest that the altered chromatin structure caused by the HAT activity of p300 may have an important role for the Sox9-dependent transcription.

Sox9-dependent Transcription Is Regulated by p300-mediated Histone Acetylation on Chromatin—Next, we explored the relationship between p300-induced histone acetylation and Sox9-dependent transcriptional activation on naked or chromatinized DNA. Because p300-mediated acetylation requires Acetyl co-A as a substrate, we performed in vitro transcription assays in the presence and absence of Acetyl co-A. The addition of Acetyl-CoA had no significant effect on in vitro transcription of a naked DNA template (Fig. 4B, lanes 1–5). In contrast, the absence of Acetyl-CoA severely reduced Sox9-dependent transcription

FIGURE 1. The differences between human MSCs and chondrocytes in expression of Col2a1, Sox9, and p300. Each sample was prepared from individual donors. The expression of type II collagen in chondrocytes was confirmed by reverse transcription PCR (A) and Western blotting analyses (B and C). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) signals were used as internal controls. Equal amounts of whole cell lysates (40 μg) and nuclear extracts (20 μg) were loaded on 8% SDS-polyacrylamide gels (panels B and C, respectively). The nuclear extracts of chondrocytes contained higher amounts of Sox9 and p300 than those of MSCs (C). Protein levels were then normalized using RNA polymerase II signals.

FIGURE 2. Sox9-dependent protein complex formation in nuclear extracts. Immunoprecipitated recombinant Sox9 (amount equal to 20 ng) purified with anti-FLAG M2 affinity gels was loaded as a control (lanes 1 and 4). Nuclear extracts (50 μg) of chondrocytes or SW1353 cells were mixed with affinity gel-conjugated Sox9 (50 ng), and coinmunoprecipitated fractions were analyzed on 8% SDS-polyacrylamide gels (lanes 2 and 3). As a control, immunoprecipitation was performed with nuclear extracts (50 μg) and anti-FLAG affinity gels (lanes 3 and 6). p300, which is contained in the nuclear extracts of chondrocytes or SW1353 cells, was co-immunoprecipitated with recombinant Sox9 (blotted with anti-p300 antibodies). Smad2/3 and PGC-1α were also detected as Sox9-associated molecules in the nuclear extracts.
after chromatin assembly (Fig. 4B, lanes 6–8). Additionally, p300 failed to stimulate the Sox9-dependent transcription in the absence of Acetyl-CoA (Fig. 4B, lanes 9 and 10). In HAT assays, free histones were acetylated by p300 even in the absence of Sox9 (Fig. 4C, section labeled Chromatin). However, both p300 and Sox9 synergistically acetylated histones, which were assembled on a chromatin template (Fig. 4C, section labeled Chromatin). These findings suggest that Sox9-regulated gene expression and subsequent chondrogenesis might be induced by chromatin acetylation through p300 recruitment by Sox9.

Histone Hyperacetylation Activates Cartilage Matrix Gene Expression in Chondrocytes—To assess the relationship between COL2A1 expression and histone acetylation on the COL2A1 enhancer region, we used the histone deacetylase inhibitor TSA to induce histone hyperacetylation in human chondrocytes. TSA treatments stimulated COL2A1 expression in chondrocytes (Fig. 5A). The expression of Aggre-
can, one of the cartilage-specific matrix genes regulated by Sox9, was also enhanced by TSA treatments (Fig. 5B). Chromatin immunoprecipitation assays revealed that histones H3 and H4 around the COL2A1 enhancer region were highly acetylated by TSA treatments (Fig. 5C). These results also suggest that Sox9-dependent transcription might be activated by histone acetylation during chondrocyte differentiation and maturation.

**DISCUSSION**

The present study demonstrates that the p300 HAT activity modulates the Sox9-dependent transcription on the assembled chromatin. Our findings suggest that p300-mediated chromatin modifications are important steps for the induction of chondrogenesis (21–28,38). The transcriptional regulatory property of p300 is exerted through several mechanisms. p300 acts as a protein scaffold and bridging factor for forming multicomponent complexes and connecting DNA-binding transcription factors to the transcription apparatus. In addition, the intrinsic HAT activity of p300 has the potential to facilitate the transcriptional activation by histone modification (23, 24, 38). However, it is not fully known if p300-HAT activity regulates transcription by altering chromatin structure during chondrogenesis.

In this report we investigated the function of p300 in Sox9-dependent transcription using an *in vitro* chromatin assembly model. Several reports have shown the importance of p300-mediated chromatin remodeling activity for transcriptional activation by using chromatin reconstitution models (20–22, 25, 26, 39). The HAT activity of p300 also plays a critical role for the activation of CREB-, MyoD-, p53-, and vitamin D receptor-dependent transcription on reconstituted chromatin (20, 21, 25, 26). In our chromatin assembly assays, p300 stimulated Sox9-dependent transcription through the modification of histone acetylation (Fig. 4, B and C). These findings suggest that p300-dependent histone acetylation regulates chondrogenesis.

Animal models have revealed a fundamental role for p300 in embryonic development. The *p300* (−/−) and *p300* (+/−) mice show embryonic lethal phenotypes (40). Double heterozygosity for *p300* and *Cbp* also results in early lethality (40). In addition, *Cbp* heterozygous mice and Rubinstein-Taybi syndrome (a heterozygous mutation in *CBP* allele) show skeletal abnormalities (41, 42). From these observations, embryogenesis and cellular differentiation might be regulated by gene dosage and overlapping roles of p300 and CBP. Consistent with this hypothesis, we observed that the amount of p300 in multi-potential human MSCs was lower than that in human chondrocytes (Fig. 1C). In vitro immunoprecipitation analyses showed purified Sox9 associated with p300 in chondrogenic cells (Fig. 2, sections labeled Chondrocytes and SW1353 cells) but not in MSCs (data not shown). Because the Sox9-regulated *Col2a1* expression is increased by p300 (8, 10), our findings suggest that gene dosage-dependent regulation by p300 might modulate chondrogenesis through association with Sox9.

We demonstrated previously that Smad3 and PGC-1α regulate *Col2a1* expression by associating with Sox9 and p300 (9, 10). In this study, purified Sox9 formed a protein complex with endogenous Smad3, PGC-1α, and p300 *in vitro* (Fig. 2). Recent observations support the idea that transcription is regulated by multi-protein complexes that associate with DNA-binding transcription factors (26, 43). To understand the precise mechanism of Sox9-dependent gene expression, it would be useful to purify the whole Sox9-related molecular complex from chondrocytes and analyze its function on chromatin templates.

Cartilage matrix proteins have important roles in the maintenance and organization of chondrocyte phenotypes. The presence of cartilage-specific matrix proteins in the cell signify the developmental stages of chondrogenesis. Many of these are regulated by the sequence-specific transcription factor Sox9 (6, 44–47). The expression of the cartilage oligomeric matrix protein gene is down-regulated by histone deacetylase-1 but increased by the histone deacetylase inhibitor TSA (48), indicating that histone acetylation is involved in gene expression. However, there are few reports that describe the functional link between histone acetylation and Sox9-regulated gene expression in chondrogenesis. In this study, we demonstrated that TSA treatments enhanced the expression of *COL2A1* and *Aggrecan* in chondrocytes (Fig. 5, A and B) and also increased the acetylation levels of histone H3/H4 around the COL2A1 enhancer region containing the Sox9-binding site (Fig. 5C). In contrast, no significant differences in the expression of *COL2A1* and *Aggrecan* in human MSCs was observed after TSA treatments (data not shown). These findings indicate that TSA-induced histone hyperacetylation stimulates Sox9-regulated gene expression and support our findings that p300-mediated histone acetylation promotes Sox9-dependent transcription and chondrogenesis. Further investigation of the switching mechanism between histone deacetylase and p300 and the effect on Sox9 may provide a novel therapeutic target for cartilaginous diseases, including osteoarthritis.

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