Sox9-dependent transcriptional regulation of the proprotein convertase furin

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Guimont P, Grondin F, Dubois CM. Sox9-dependent transcriptional regulation of the proprotein convertase furin. Am J Physiol Cell Physiol 293: C172–C183, 2007. First published March 14, 2007; doi:10.1152/ajpcell.00349.2006.—The proprotein convertase furin participates in the maturation/bioactivation of a variety of proproteins involved in chondrogenesis events. These include parathyroid hormone–related peptide (PTHrP), an autocrine/paracrine factor that is crucial to both normal cartilage development and cartilage-related pathological processes. Despite the known importance of furin activity in the bioactivation of the polypeptides, the mechanisms that control furin regulation in chondrogenesis remain unknown. To gain insight into the molecular regulation of furin, we used the mouse prechondrogenic ATDC5 cell line, an established in vitro model of cartilage differentiation. Peak expression of both furin mRNA and furin PTHrP maturation was observed during chondrocyte nodule formation stage, an event that correlated with increased mRNA levels of Sox9, a potent high-mobility-group (HMG) box-containing transcription factor required for cartilage formation. Inhibition of furin activity led to a diminution in maturation of PTHrP, suggesting a relationship between Sox9-induced regulation of furin and chondrogenesis events. Transient transfection of Sox9 in nonchondrogenic cells resulted in a marked increase in furin mRNA and in the transactivation of the furin P1A promoter. Direct Sox9 action on the P1A promoter was narrowed down to a critical paired site with Sox9 binding capability in vitro and in vivo. Sox9 transactivation effect was inhibited by L-Sox5 and Sox-6, two Sox9 homologs also expressed in ATDC5 cells. Sox6 inhibitory effect was reduced when using Sox6-HMG-box mutants, indicating a repressive effect through direct HMG-box/DNA binding. Our work suggests a mechanism by which furin is regulated during chondrogenesis. It also adds to the complexity of Sox molecule interaction during gene regulation.

CARTILAGE IS A COMPLEX TISSUE containing a unique set of extracellular matrix (ECM) molecules that provides the tissue its ability to withstand compression and frictional stress associated with joint movement (44). Chondrocytes, at different stages of differentiation, are responsible for the precise production of ECM in several different types of cartilage tissue in the developing vertebrate, including growth plate cartilage and articular cartilage (55). The differentiation of mesenchymal cells into chondrocytes occurs along a complex differentiation pathway involving coordinate expression of growth factors and receptors as well as signal transduction molecules.

Many of these molecules are synthesized as proproteins and require proteolytic processing to generate functional peptide products. For example, the parathyroid hormone-related peptide (PTHrP) is essential for the transition from the prehypertrophic to hypertrophic stage of chondrogenesis (28, 63). Both PTHrP and PTHrP receptor (PPR) null mice show premature mineralization and ossification that appear to be related to the premature transition of proliferative to hypertrophic chondrocytes (29, 63). Members of the transforming growth factor (TGF)-β family, including TGFβs and bone morphogenetic proteins (BMPs), are essential for proper chondrocyte differentiation and production of cartilage matrix. The loss of responsiveness to TGFβ in mice promotes terminal differentiation of chondrocytes and results in degenerative joint disease resembling osteoarthritis (53, 68).

These polypeptides are substrates for the proprotein convertase (PC) furin (8, 14, 18, 39). This convertase is an essential endoprotease of the constitutive secretory pathway that activates a variety of precursor proteins through proteolysis at the COOH-terminal side of the consensus sequence Arg-X-Lys/Arg-Arg (50, 60). Furin has a critical role in developmental processes, and increasing evidence suggests that this protease plays a key role in cartilage formation. By midgestation stages, furin is abundantly expressed in all precartilaginous bodies in skeletogenic centers, such as the cartilage primordium of cephalic bones, vertebral bodies, ribs, and femurs (70). In normal articular cartilage, immunoreactive furin is almost exclusively expressed in chondrocytes of the superficial zone, whereas in osteoarthritic cartilage, furin expression is modified and detected in articular cells throughout the cartilage tissue (43). Thus furin, at appropriate localization and levels, seems to be important for both the development and the maintenance of normal cartilage. To date, however, the mechanisms that control furin gene expression in chondrocytes remain unknown.

A number of studies have searched for specific transcription factors that control chondrocyte differentiation. Sox (Sry-type HMG box) proteins, which form a subfamily of DNA-binding proteins that features a high-mobility-group (HMG) domain, have critical functions in a number of developmental processes, including sex determination (20, 62), neurogenesis (31, 58), and skeleton formation (5, 35, 47). The minimal consensus binding sequence for Sox proteins has been defined as the heptameric sequence [(A/T) (A/T) CAA(A/T)G] (22, 42). Analyses of the furin P1, P1A, and P1B promoter sequences reveal the presence of putative DNA recognition elements for members of the Sox transcription factors, including several closely spaced sites characteristic of Sox9-regulated genes. Among the Sox molecules, the Sox9, the long form of Sox5 (L-Sox5), and the Sox6 genes are expressed in chondrogenitor cells and have high levels of expression in chondrocytes and other cell types (7, 36). One of the earliest molecules required for cell commitment during skeletogenesis is Sox9 (7). An essential role for Sox9 in cartilage formation was demonstrated by the identification of Sox9 mutations in a patient with campomelic dysplasia (6), a disease characterized...
by severe malformations of essentially all cartilage-derived structures and associated in some cases with XY sex reversal (19, 32, 64). In mouse and human embryonic cartilages, Sox9 is expressed at various differentiation stages where it directly regulates the transcription of Col2a1 (35, 47), Col9a1 (69), Col11a1 (9, 10), and Agc (52) genes involved in cartilage matrix formation. The most convincing evidence that Sox9 is required for mesenchymal condensation comes from mouse genetics studies. In chimeric murine embryos, Sox9−/− embryonic stem (ES) cells, in contrast to wildtype ES cells, are excluded from mesenchymal condensation, indicating that Sox9 is essential for their formation (7).

The finding that furin is abundantly expressed in cartilage tissues, where this convertase is involved in the maturation/activation of several growth factors and hormones involved in crucial steps of chondrogenesis, prompted us to investigate the expression and regulation of the fur gene in chondrocytes. In this report, we demonstrate the developmental expression profile of furin in the murine prechondrogenic ATDC5 cell line, an established in vitro model of cartilage differentiation, and the involvement of Sox molecules in such regulation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and reagents.** The human fur promoter-luciferase constructs pGL2-P1, pGL2-P1A, and pGL2-P1B were generously provided by Dr. Torik A. Y. Ayoubi (University of Leuven and Flanders Interuniversity, Leuven, Belgium). The plasmids mL Sox5/pcDNA3, mSox6A/pcDNA3, hSox9/pcDNA3, and 4X48p89luc were kindly given by Dr. Benoit de Crombrugghe (University of Texas M. D. Anderson Cancer Center, Houston, TX). All mSox6A HMG domain mutants were produced in our facility by site-directed mutagenesis using the Quick Change Mutagenesis kit (Stratagene, Cedar Creek, TX) and sequenced. For EMSA, cartridge purity-grade oligonucleotides, (Gibco, Burlington, ON, Canada) were used. Phenylmethylsulfonyl fluoride (PMSF), leupeptin, and pepstatin A were from Sigma (Oakville, ON, Canada); poly(dI-dC) was from Amersham Biosciences (Baie d’Urfe, QC, Canada); T4 polynucleotide kinase was from New England Biolabs (Pickering, ON, Canada); aprotinin and Nonidet P-40 (NP-40) were from Roche (Mississauga ON, Canada); [γ-32P]ATP was from PerkinElmer (Woodbridge, ON, Canada); and acetylated BSA was from Promega (Mississauga, ON, Canada).

**Cell cultures.** ATDC5 cells, generically provided by Dr. Y. Yamada (National Institutes of Health, Bethesda, MD), are clonal mouse carcinoma cells derived from the AT805 cell line (3). They display characteristics of committed chondroprogenitor cells and undergo insulin-induced differentiation that mimics chondrocyte differentiation in vivo (54). ATDC5 cells were maintained in DMEM and Ham’s F-12 (DMEM/F-12; Gibco) medium containing 5% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Wisent, St-Bruno, QC, Canada), 10 μg/ml human transferrin (T; Gibco), and 30 nM sodium selenite (S; Sigma). For chondrocyte differentiation, ATDC5 cells were seeded at 4×104 cells in 10-cm petri dishes (Sarstedt, Montreal, QC, Canada) at 37°C under 5% CO2, and 10 μg/ml bovine insulin (I; Sigma) were added to the complete supplemented DMEM/F-12 medium. On day 21, the culture medium was changed to αMEM (Gibco) with 5% FBS containing the same supplements (56), and the CO2 concentration was diminished to 3%. αMEM contains ascorbic acid in the standard formula to facilitate the induction of the hypertrophic phenotype in these cells (54). The medium was changed every other day for 28 days. Cos7 cells [American Type Culture Collection (ATCC), Rockville, MD] were maintained in DMEM with high glucose containing 10% FBS (Gibco) and 40 μg/ml garamycin (Sabex, Boucherville, QC, Canada). HepG2 cells (ATCC) were maintained in MEM (Gibco) containing 10% FBS and 40 μg/ml garamycin.

**Luciferase assays.** HepG2 or Cos7 cells were transiently transfected by the CaPO4 precipitation technique using a mammalian cell transfection kit (Speciality Media, Lavale, NJ), as previously described (33). Briefly, 24 h before transfection, HepG2 and Cos7 cells were plated into six-well plates (Falcon Labware, Mississauga, ON, Canada) at a density of 125,000 and 100,000 cells/well, respectively. The cells were fed with fresh complete medium 3–4 h before transfection. Cells were transfected with 2 μg DNA/well for each plasmid, according to results from dose-response studies. Control vectors were used to compensate for potential squelching. These vectors were tested separately to ensure that they do not respond to the treatment. After overnight incubation, the cells were lysed, and luciferase activity was measured. The results were expressed as fold-induction of luciferase activity.

**Fig. 1.** Furin and Sox expressions in differentiating ATDC5 cells. Cells were cultured in DMEM/F-12 containing 5% FBS and insulin-transferrin-sodium selenite (ITS) for various time periods, as indicated, and total RNA was extracted. mRNA levels were measured by real-time PCR, normalized to 18S, and 40 μg/ml bovine insulin (I; Sigma) were added to the complete supplemented DMEM/F-12 medium. On day 21, the culture medium was changed to αMEM (Gibco) with 5% FBS containing the same supplements (56), and the CO2 concentration was diminished to 3%. αMEM contains ascorbic acid in the standard formula to facilitate the induction of the hypertrophic phenotype in these cells (54). The medium was changed every other day for 28 days. Cos7 cells [American Type Culture Collection (ATCC), Rockville, MD] were maintained in DMEM with high glucose containing 10% FBS (Gibco) and 40 μg/ml garamycin (Sabex, Boucherville, QC, Canada). HepG2 cells were maintained in MEM (Gibco) containing 10% FBS and 40 μg/ml garamycin.
Semiquantitative RT-PCR. ATDC5 cells were grown for up to 28 days, and total RNA was extracted according to the Trizol reagent protocol (Invitrogen, Burlington, ON, Canada). Gene expression was analyzed by semiquantitative RT-PCR; 2.5 μg of total RNA were reverse transcribed in a 20-μl reaction using 2 μl of random decamers (Ambion, Austin, TX) and 200 U of Superscript II RT (Invitrogen). PCR was performed in a 50-μl volume containing 1 μl of the cDNA product, 1 μM gene-specific primers, and 1.25 U of Taq DNA polymerase (Roche). For each primer, the melting temperature was carefully determined to obtain only the DNA band of interest. Reaction products were analyzed on 1.5% agarose gels in the presence of 2.7 μg/ml ethidium bromide (Sigma), and a digital image of each gel was captured using a gel documentation system (Kodak digital science DC120 camera and software).

The sequences of the specific pairs of primers used for PCR were as follows: sense 5'-CGAAGACGAGAGCCAAGGGACGTCGTA-3' and antisense 5'-CAAGTCACCTCGGCCATCAACATC-3' for furin, sense 5'-GAAGAGCCAAGGGACGACGACATGAGGTA-3' and antisense 5'-AGGTCAAGGAGAGAGAGAGGTA-3' for TGF-β1, sense 5'-CAAGGTCACTCCAAGACTGTCC-3' and antisense 5'-TGTTGCTTGTGAAGAGATGTCC-3' for TGF-β1, sense 5'-GGTTACACCTCGGCCATCAACATC-3' and antisense 5'-TGTGCTTGTGAAGAGATGTCC-3' for TGF-β3, sense 5'-GGGTTCGGG-3' and antisense 5'-GGATTGTGTTTCA-3' for L-Sox5, sense 5'-CAGGAAAAGAGAATCCATTGTG-3' and antisense 5'-TCTCTTCGATCGTCTCCATCTC-3' for L-Sox5, sense 5'-CAAGGTCAAGAGATCCATCCTCTC-3' and antisense 5'-ATCCATTGTGTTTGTTTCTT-3' for Sox6, sense 5'-GGGGAAGGCTGGGCAAGG-3' and antisense 5'-CACACTGTTAGTGGGCAAGG-3' for Sox9, sense 5'-CACACTGTTAGTGGGCAAGG-3' and antisense 5'-CACACTGTTAGTGGGCAAGG-3' for Sox9.

Real-time PCR. fur and 18S DNA were cloned in pGEMT plasmid (Promega) using the Promega protocol. Both plasmids were amplified in bacteria, extracted with the Qiagen midiprep plasmid extraction kit (Qiagen), and diluted from 10^9 to 10^7 copies/μl to perform standard curve amplification. The reaction mixtures for real-time PCR were optimized for the Rotor-Gene 3000 (Corbett Research, Kirkland, QC, Canada) and consisted of 2.5 mM MgCl2, 1.6 μl of SYBR Green (1/1,000), and 0.5 μl of reverse transcription reaction product in a final volume of 25 μl. The cycling program consisted of an initial denaturation at 95°C for 5 min, 45 cycles of amplification with an annealing temperature of 60°C for 18S, 29 cycles at 60°C for furin, 40 cycles at 56°C for TGFβ1, 38 cycles at 62°C for PTHrP, 35 cycles at 68°C for TACE, 40 cycles at 58°C for L-Sox5 and Sox6, 30 cycles at 55°C for Sox9, 32 cycles at 60°C for furin, 34 cycles for type II collagen, 34 cycles at 60°C for type X collagen, and 25 cycles at 60°C for 18S.

Fig. 2. Sox9 transactivates the human fur gene promoter in Cos7 and HepG2 cells. A: Cos7 cells were transfected with pcDNA3-hSox9, and furin mRNA levels were measured by real-time PCR, normalized to 18S ribosomal RNA, and quantified as fold-induction relative to the activity of fur promoters co-transfected with control vectors. Data are expressed as means ± SE; n = 5. Inset: HepG2 cells were transfected with pcDNA3-hSox9 or with empty vector. Whole cell lysates and nuclear extracts were analyzed by Western blotting using anti-Sox9 antibodies.
bation. 10 µg of nuclear extracts, prepared as described (40), were analyzed by EMSA (16). Protein-DNA binding reactions were carried out with 40 000 cpm oligonucleotide probe (32P end-labeled with T4 kinase) in Sox9-binding buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 10% (vol/vol) NP-40] supplemented with 30 µg of acetylated BSA and 0.75 µg of poly(dI-dC) before addition of the labeled probe (15 min, room temperature). For supershift experiments, binding reactions were conducted in the presence of Sox9-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or isotype-matched control antibodies (Sigma) (30 min on ice) before addition of the 32P-labeled probes. The sequences of the sense strands of the oligonucleotides used for EMSA were as follows: 5’-CCAGCATTGTCTCCAGTCCAGT-3’ (P1A AB sites), 5’-CCAGTTAAAAAGGCTTTTTAAGAGAAGAGAGAGAAG-3’ [P1A AB site mutant (mut)], 5’-CCAGTTAAAAAGGCTTCAAGTCTCCAGTCCAGT-3’ (P1A A site mut), 5’-CCAGCATTGTCTC-CTTTAAAAAACAGTT-3’ (P1A B site mut), 5’-GGTTTGTCAAGGCCCTTGTGGCC-3’ (SOLL11a2) (10).

Western blot analysis. Total cell lysates, nuclear extracts, and immunoblotting were performed as previously described (8). The membranes were probed overnight with either rabbit anti-Sox9, anti-Sox6, or anti-PTHrP antibodies (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated anti-rabbit IgG (Sigma). Blots were developed using enhanced chemiluminescence Western blotting detection reagent (Amersham). In selected experiments, the furin inhibitor dec-RVKR-cmk (Bachem, Torrance, CA) or hexa-arginine (Calbiochem) was added 24 h before the end of cultures.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assays were performed using the EZ ChIP assay kit (Upstate, Lake Placid, NY) according to the manufacturer’s protocol. HepG2 or ATDC5 samples were immunoprecipitated with either Sox9-specific antibodies (Santa Cruz Biotechnology) or isotype-matching rabbit IgG (Sigma). Primer sequences for amplification of the distal ( Sox sites-containing) region of human or corresponding mouse P1A promoter were as follows: 5’-CGAGAAGTCTCATGTG-3’ and 5’-TGTGGCCCCTAG-3’ (antisense) for the human sequence, with a predicted amplicon size of 218 bp, and 5’-CCAGCATTGTTCCTCCAGTTA-3’ (sense) and 5’-CCACATAATCCGCTCAGGATGACA-3’ (antisense) for the mouse sequence, with a predicted amplicon size of 252 bp.

RESULTS

Modulated expression of furin in differentiating ATDC5 cells. To gain insight into the regulation of the fur gene (encoding furin) during chondrogenesis, we used the mouse prechondrogenic ATDC5 cell line, an established insulin-induced in vitro model of cartilage differentiation that displays the entire spectrum of chondrocyte differentiation (54). As demonstrated in Fig. 1A, collagen type 2α1 mRNA was expressed in ATDC5 cells from day 3 onward, concomitant with the cells being elongated and forming cellular condensation (1). Gene expression for the type 10α1 collagen was detectable during both prehypertrophic and hypertrophic stages (days 21–28) (54). In addition, Sox 9 mRNA was first detected (faint band) at the precondensation stage (day 3), with peak expression at the nodule formation stage (days 7–14), followed by a gradual decline in expression levels during the prehypertrophic (day 21) and hypertrophic (day 28) stages. In contrast, L-Sox5 and Sox6 mRNAs were expressed throughout the ATDC5 differentiation process. The observed pattern of expression of the chondrogenic differentiation markers collagen type 2α1, collagen type 10α1, and Sox9 is in agreement with previously published data (1, 3, 54).

Analysis of furin mRNA levels revealed an expression pattern similar to the one observed for Sox9, with absence of mRNA detection at the precondensation stage, peak levels at the nodule formation stage, and a gradual decline thereafter. The furin substrates PTHrP and TGFβ1 (17, 18, 39) closely followed the furin expression pattern. As a control, TACE/ADAM17, a less restrictive furin substrate (59), was expressed throughout the differentiation process. Real-time PCR analysis

Fig. 3. Basal and Sox9-induced transactivation of P1A 5’s deletion constructs. A: schematic representation of consensus Sox recognition sequences within the fur P1A promoter fragments shortened in 5’s using endogenous Stud, AgeI, Msel, and NotI restriction sites. Positions are relative to the 3’s end of P1A promoter sequence. B and C: HepG2 cells were co-transfected with either 2 µg of pGL2-P1A or each of the 5’s deletion constructs in the presence of pcDNA3-Sox9 (8) or in the presence of empty pcDNA3 (C). After 48 h, basal and Sox9-induced luciferase activities were determined and expressed as relative luciferase units (RLU). Data are expressed as means ± SE; n = 8. *P < 0.05 and ***P < 0.0001 by unpaired Student’s test.
was performed to quantitatively measure furin mRNA expression levels. As observed in Fig. 1B, the number of furin gene copies detected correlated with the expression pattern obtained with the semiquantitative PCR assay (Fig. 1A). Taken together, our results indicate that ATDC5 cell cultures differentiate in a time-dependent fashion in the presence of insulin, as previously reported. They also indicate that the expression of furin and Sox9 genes are co-modulated during the ATDC5 differentiation process, suggesting a relationship between Sox9 and furin expression.

Sox9 acts to amplify fur gene expression. To determine whether Sox9 modulates endogenous fur gene expression, we used Cos7 cells, which were shown to display undetectable levels of endogenous Sox9 (data not shown). These cells were transfected with human Sox9 expression vector (pcDNA3-hSox9) followed by real-time PCR analysis of fur transcripts. As indicated in Fig. 2A, Sox9 expression resulted in a 4.3-fold increase in furin mRNA levels. Thus Sox9 positively regulates fur gene expression in cells.

Differential sensitivity of the fur promoters to the transcription factor Sox9. Previous reports have demonstrated that fur transcription is regulated by three promoters, namely P1, P1A, and P1B (4). To determine whether Sox9 directly influences furin transcription, we measured the effect of Sox9 expression on the transcriptional activity of each of the three furin promoters in HepG2 cells (depicted in Fig. 2B). This cell line has been used previously to study the impact of various transcription factors/signaling molecules on furin gene expression (8). The HepG2 cell line was used instead of the ATDC5 cells because it is easier to transfect, and even though these are nonchondrogenic cells, they express basal levels of Sox9 protein (Fig. 2B, inset). For furin promoter assays, HepG2 cells were transiently transfected with the furin luciferase reporter constructs pGL2-P1, pGL2-P1A, and pGL2-P1B or the Col2a1 reporter construct 4X48p89Col2a1 in the presence of pcDNA3-hSox9 or an empty vector as control. In cells transfected with Sox9, whereas the activity of the P1 or the P1B promoters was not upregulated, an approximately 5-fold increase in P1A transactivation was observed (Fig. 2B). The response of the P1A promoter to Sox9 was similar to the one obtained with the col2a1 reporter construct containing four tandem repeats of the 48-bp Col2a1 promoter, a strong chondrocyte-specific enhancer known to be activated by Sox9 (37). These results suggested that the fur gene is transactivated by Sox9 and that strong Sox regulatory regions reside within the furin P1A promoter.

Deletion analysis of the furin P1A promoter. To delineate the P1A promoter region(s) implicated in Sox9 regulation, 5' deletion constructs (shown in Fig. 3A) were co-transfected into HepG2 cells along with the pcDNA3-hSox9 expression vector. As shown in Fig. 3, B and C, both basal and Sox9-induced P1A responses were retained up to position -2096. Further dele-

Fig. 4. Functionality of Sox heptameric sequences within P1A promoter. A: nucleotide sequences of the putative Sox sites within the fur P1A promoter. Positions are relative to the 3' end of P1A. The 3 putative SOX-binding sites are designated A–C. The sequences of the mutated fragments are shown at bottom. Only mutated nucleotides are indicated; unchanged nucleotides are represented by dots. B: HepG2 cells were co-transfected with 2 μg of wildtype (WT) or Sox-mutated (mut) pGL2-P1A constructs and 2 μg of pcDNA3-hSox9 or empty plasmid as control. Luciferase activities are expressed as fold relative to pGL2WT values set at 1. Data are presented as means ± SE; n = 3. ***P < 0.001 and ****P < 0.0001 by unpaired Student's test.
tions to position −1563 resulted in a sharp decrease in basal (Fig. 3B) or Sox9-mediated activity (Fig. 3C) capabilities near to values obtained with the basic pGL2 promoter. We therefore conclude that Sox9-responsive elements reside within the −2096 to −1563 promoter region of the P1A furin promoter.

Functional analysis of Sox9-binding sites within the −2096 to −1563 P1A promoter sequence. The common Sox9-binding site was previously shown to consist of a heptamer AT-rich DNA sequence, although recent data highlighted the presence of a more complex architectural binding sequence within the 5′-UTR of several Sox9-induced genes. Such a motif consists of two Sox recognition sites, separated by three or four nucleotides, that face each other to allow the binding of Sox9 as a dimer (10). Analysis of the Sox9-sensitive −2096 to −1563 promoter sequence revealed the presence of three putative Sox recognition sites, designated A, B, and C (Fig. 4A). Sites A and B have features of a paired Sox site in that they are oriented toward each other and spaced by four nucleotides, whereas the C site harbors characteristics of a single heptamer Sox sequence located 255 bp downstream from the A-B site. To define more precisely the role of these putative Sox-binding sites in P1A activity, point mutations were introduced into sites A, B, and C, resulting in four distinct mutants: pGL2-P1A-mutA, pGL2-P1A-mutB, pGL2-P1A-mutAB, and pGL2-P1A-mutC (Fig. 4A). Mutations of site A and more importantly site B decreased Sox9-induced promoter activity, while mutations of both A and B sites resulted in only slight but nonsignificant additional inhibition, suggesting that these two sites act in a cooperative manner (Fig. 4B). In contrast, mutation of the C site did not significantly reduce Sox9 activity, further suggesting that this site is dispensable for the Sox9-induced response of the P1A promoter.

To determine whether the tandem Sox sequence (A and B sites) could bind Sox9, we performed EMSA with a 27-bp probe containing both wildtype A and B sites (oligonucleotide P1A-AB, Fig. 5A). Three retarded bands (C1, C2, and C3) were obtained with nuclear extract from Sox9-overexpressing HepG2 cells (Fig. 5B). All three complexes were effectively competed using a cold oligonucleotide featuring a consensus Sox9 sequence (10) (data not shown). In contrast, mutated oligonucleotides with 3-bp substitutions within the A and B motifs (P1A-AB mut) were unable to form complexes (Fig. 5, lanes 4–6). In addition, a Sox9-specific antibody supershifted into two slower migrating bands named the C3, C2, and C1 complexes (Fig. 5B, lane 3), which correspond to monomeric Sox9, dimeric Sox9, and Sox9-binding factor complexes, respectively (2, 6, 10, 15, 27, 57), whereas an isotype-matched control antibody was inefficient (Fig. 5, lanes 4–6). These observations indicate that Sox9 is a component of the C1, C2, and C3 complexes. Taken together, these results show that the Sox consensus sequences located between positions −1856 and −1814 of the furin P1A promoter specifically interact with Sox9.

To study in more detail the relative contribution of site A and site B in Sox 9-binding activity, the A and B sites of the P1A-AB oligonucleotide were individually mutated. As demonstrated in Fig. 5B, disruption of the A site or its inversion (P1A-Amut; lanes 7–9 and data not shown) resulted in the formation of only a high-mobility complex, corresponding to the monomeric Sox9 DNA complex (C3), whereas mutations in either the B site (P1A-Bmut; lanes 10–12) or both A and B sites (P1A-ABmut; lanes 4–6) prevented the formation of all three Sox9 complexes. These results suggest that Sox9 binds to the B site as a monomeric form, whereas the A site does not bind any Sox9 complexes. In addition, and according to previous reports (48), they indicate that both A and B sites act in a cooperative manner for the binding of the higher-molecular-weight C2 (dimeric Sox9) and C1 (Sox9-binding factor) complexes.

Chromatin DNA binding of endogenous Sox9 in HepG2 and ATDC5 cells. The nucleotide sequences of the mouse and human furin exon 1A (P1A promoter) are highly similar (90% identity between them), suggesting that this untranslated exon might have some important and specific function(s) that has been conserved during evolution (4). Supporting this, the A and B Sox sites are conserved within the mouse P1A promoter, with two mismatches within the mouse and human A site
(cttttgtg) and 100% homology for the B site (cagttcc). To confirm that Sox9 directly binds to the tandem P1A A/B Sox site, ChIP assays were performed in both human HepG2 and murine ATDC5 cells. Cross-linked cell lysates were immunoprecipitated with anti-Sox9 or control antibodies, and PCR was used to amplify a 218- or 252-bp fragment of P1A promoter using primers flanking the human or murine A/B site, respectively. The results revealed that, while Sox9 constitutively binds to the human P1A Sox site in HepG2 cells, 7 days of differentiation are needed for high-level binding of this transcription factor to the homologous ATDC5 P1A site (Fig. 5C). This observation is in line with the demonstration that Sox9 is constitutively expressed in human HepG2 cells, while substantial levels of Sox9 are observed only on ATDC5 differentiation.

L-Sox5 and Sox6 act as negative regulators of Sox9-induced fur expression. It has been demonstrated that Sox9 cooperates with L-Sox5 and Sox6 to regulate the expression of various genes, including type II collagen and cartilage link protein (CRTLI) (26, 30, 36). To provide insight into the possible participation of Sox6 and L-Sox5 proteins in the regulation of the fur gene, endogenous Sox9 expressing HepG2 cells or Sox9-negative Cos7 cells were co-transfected with pGL2-P1A and the three Sox-encoding plasmids (pCDNA3-L-Sox5, pCDNA3-Sox6, pCDNA3-Sox9), alone or in combination, and luciferase activity was measured. As shown in Fig. 6A, expression in HepG2 cells of L-Sox5 and Sox6 alone or in combination resulted in an 18.2, 57.3, and 70.4% reduction, respectively, of basal P1A transcriptional activity. This effect is statistically significant with Sox6 alone or L-Sox5 and Sox6 combinations. Such basal inhibitory impact of L-Sox5, Sox6, or their combination was not observed with the Sox9-negative cell line Cos7 (Fig. 6B), suggesting the requirement of an interaction between L-Sox5 and/or Sox6 and endogenously expressed Sox9 in HepG2 cells for the inhibition of P1A activity. Supporting this, co-transfection of L-Sox5 and/or Sox6 with Sox9 abolished Sox9-induced P1A transactivation in both HepG2 and Cos7 cells (Fig. 6, A and B). Data from real-time PCR analysis of fur transcripts in Cos7 cells demonstrated a similar action of L-Sox5 and Sox6 in reducing Sox9-induced expression of furin mRNA (Fig. 6C). Taken together, these results indicate a negative cooperation among L-Sox5, Sox6, and Sox9 for both furin P1A transactivation and furin mRNA expression.

It is known that Sox DNA binding requires the presence of highly conserved amino acids within the HMG domain (25). In particular, phenylalanine, at position 12, was shown to be a key residue within the hydrophobic core that interacts with the Sox consensus DNA sequences. To define whether the negative cooperation between Sox6 and Sox9 involves direct binding of Sox6 to DNA, we tested the impact of two previously characterized Sox-HMG-box mutants, namely Sox6F12Y and Sox6Y74A, on Sox9-induced transactivation (Fig. 7A). Previous characterization of these mutants indicated that Sox6F12Y and Sox6Y74A exhibited 1.8 and 44.5% DNA-
binding capacity to a consensus Sox motif compared with Sox6wt (25). Co-transfection of various concentrations of Sox6wt-encoding plasmid together with Sox9 resulted in a concentration-dependent inhibition of Sox9-induced P1A transactivation, with a 94% inhibition obtained with the highest concentration of plasmid (4 μg) used. In contrast, the Sox6F12Y mutant, which displays very low DNA-binding capacity, failed to diminish Sox9-induced P1A transactivation, whereas the intermediate DNA-binding affinity mutant, Sox6Y74A, had a milder impact, with 71% inhibition achieved (Fig. 7B). From these results, one can conclude that the repressive effect of Sox6 requires direct binding of its HMG box to DNA.

**DISCUSSION**

The mammalian convertase furin is responsible for the proteolytic activation/maturation of critical chondrogenesis mediators such as PTHrP, BMPs, insulin-like growth factor receptor, and TGFβ, which are synthesized by chondrocytes. To date, however, data on the expression/regulation of the fur gene in these cells are lacking. We demonstrated herein that, during differentiation of the chondrogenic cell line ATDC5, fur mRNA was expressed in a similar pattern as Sox9 mRNA. Both genes showed peak expression during the nodule formation stage, with a gradual decline at the prehypertrophic and hypertrophic stages, where the expression of both Sox9 and fur mRNA was barely detectable. We also showed that the distal region of the fur P1A promoter is transactivated by Sox9, and that this transactivation depends on a paired Sox site located within the distal region of the promoter. Our work provides a mechanism by which furin is regulated during chondrogenesis and adds furin to the growing list of genes modulated by Sox9, a key regulator of chondrogenesis.

Real-time PCR results have shown that Sox9 overexpression positively regulates furin mRNA levels. As described by Ayoubi et al. (4), three alternative promoters direct fur expression. Promoter P1 was described as being of the inducible type. It has both TATA and CCAAT elements in the proximal promoter region and was reported to be trans-activated by the transcription factors C/EBP, GATA-1/FOG, SMADs, and HIF-1 (8, 34, 41). This feature is relevant to the inducible nature of the P1 promoter and to its relatively high levels of
expression in hepatocytes and during megakaryocyte development (4, 34). On the other hand, P1A and P1B promoters possess known characteristics of housekeeping gene promoters, as they are GC rich and contain several Sp1-binding sites. So far, the exact function of these two promoters remains unknown. Herein, we demonstrate that, among the three furin promoters, only P1A is transactivated by Sox9, suggesting for the first time that one of the roles of the P1A promoter is to drive furin expression during Sox9-regulated developmental processes such as cartilage/bone formation.

Promoter deletion and sequence analysis revealed three putative heptameric Sox sequences (designated A to C sites) within the Sox9-sensitive P1A promoter region. Further study of the Sox9-inducible P1A promoter indicated that point mutations within the A and B sites abolished responsiveness to Sox9, whereas mutations within the downstream C site had no impact, indicating that P1A responsiveness to Sox9 relies on a unique combination of functional elements. The presence of both A and B sites is necessary for the transcriptional activation of the P1A promoter by Sox9.

The observation that Sox9 must function as a dimer to transactivate the fur gene is consistent with published results regarding Sox10, the Sox protein member most closely related to Sox9. Both Sox10 and Sox9 have, NH2-terminal to their HMG domain, a DNA-dependent dimerization domain essential for cooperative binding to inverted paired DNA-binding sites, with spacing and orientation similar to the ones found on the furin P1A promoter.

The Sox factors L-Sox5, Sox6, and Sox9 are essential for normal skeletogenesis and cooperatively activate the expression of various genes including Col2a1 (35, 47) and matrilin-1 (49). This enhancing effect is, however, not consistent among all cartilage genes. For example, Sox9-mediated transcription of Col9a1 gene, a cartilage gene expressed at a differentiation stage other than Col2a1 or matrilin-1, is repressed by L-Sox5 and Sox6, suggesting that the regulatory mechanism of the Sox trio will differ between genes and/or differentiation stages (36, 48, 56). Results from our study indicate a negative cooperation between L-Sox5, Sox6, and Sox9 for the transactivation of the fur gene. Such repression involves direct binding of Sox6 to
DNA, since Sox6-HMG-box mutants exhibited a repressive activity that corresponds to their DNA-binding affinity. It is known that the Sox protein family members all share affinity for the same heptameric consensus sequence, (A/T)(A/T)CAA(A/T)G (22, 42). One may speculate that Sox6 and possibly L-Sox5 compete with Sox9 for binding at either one or both of the Sox sites in a pair, thereby exerting a negative regulatory effect. Results obtained from EMSA experiments using Sox6-overexpressing Cos7 cells support this possibility, since Sox6 specifically binds the Sox9 A/B enhancer element (data not shown). In addition, in the ATDC5 differentiation system, Sox9 protein levels are found increased at the nodule formation stage where peak expression of furin was detected, while Sox6 levels remained low and relatively unchanged (see supplemental materials; supplemental data are available in the online version of this article). It is therefore possible that, at this differentiation stage, the increase in Sox9 concentration will favor binding of this particular Sox family member to the fur P1A A/B enhancer.

In addition to the fur gene studies in this work, Sox9 binds as a dimer to the regulatory region of several cartilage collagen genes including Col2a1 (35, 47), Col11a2 (9, 10), Col9a2 (69), and Col27a1 (45) as well as other noncollagen cartilage genes including aggrecan (52, 57), CD-RAP (46), and matrilin-1 (49). As opposed to this, the same transcription factor was reported to bind as a monomer to cis-elements found in the regulatory region of the sex-determining genes SF1 and vanin-1 (67). The importance of Sox9 dimerization for differential regulation of Sox9-responsive sex and cartilage genes was further confirmed with the identification of patients with mutations that disrupt the dimerization domain of Sox9. These mutations resulted in a form of campomelic dysplasia characterized by cartilage abnormalities but not sex reversal, indicating that Sox9 dimerization is required for the regulation of chondrogenesis but not sex determination genes (6, 57). Consistent with this, our study indicating the requirement of a paired (A/B site) but not a single (C site) Sox site for fur regulation may explain why this enzyme is regulated during chondrogenesis while a lack of modulation was observed during testis development (46).

During rat embryogenesis, in situ hybridization studies have revealed differential spatial and temporal expression of the fur gene, with higher levels of mRNA detected in the heart and liver at early developmental stages. In mid- and late-gestational stages, furin is more widely distributed in the peripheral tissues, with a particularly abundant expression in all precartilaginous bodies of skeletogenic centers, such as the cartilage primordium of cephalic bones, vertebral bodies, ribs, and femurs (70). This pattern of embryonic expression coincides both in time and localization with the critical activity that corresponds to the chondrogenesis mediators and furin substrates PTHR1 (13), BMPs (24), and TGFβ (38, 51). It has been firmly established that PTHR1 prevents premature cartilage mineralization by inhibiting the rate at which chondrocytes, present in columns of proliferating cells, exit the cell cycle and are converted to hypertrophic chondrocytes (29, 63, 65). The critical role of PTHR1 in endochondral bone development is highlighted by the discovery of two diseases affecting the PTH/PTHrP receptor that are characterized by premature and abnormal bone mineralization and ossification (11). TGFβ and BMP-4, in turn, are essential for skeletal development, since they regulate the endochondral pathway and since BMP-4 also controls the rate of cartilage formation and chondrocyte differentiation (23, 61). In vitro, TGFβ enhances the expression of the type II collagen and aggrecan cartilage matrix protein by isolated chondrocytes as well as cartilage explants (23, 53, 61, 68). Therefore, the appropriate expression/ regulation of furin in chondrocytes would likely be important for the bioavailability of mediators critical to proper chondrocyte differentiation and cartilage matrix production.

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REFERENCES
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