SOX8 Is Expressed during Testis Differentiation in Mice and Synergizes with SF1 to Activate the Amh Promoter in Vitro

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Gonadal sex determination in eutherian mammals begins when the indifferent gonad responds to an unidentified signal resulting in the expression of Sry in males followed by the up-regulation of Sox9 expression in pre-Sertoli cells. Sertoli cells are the primary supporting cell type surrounding the germ cells and are required for further differentiation of the testes, including signaling to the steroidogenic Leydig cell lineage. One of the critical steps in male development is the regulation of expression of the anti-Müllerian hormone gene (Amh) in Sertoli cells. AMH is responsible for the regression of the Müllerian duct in males and marks the start of the hormonal cascade required for male sexual differentiation. Deletion of the Amh gene results not in full sex reversal but pseudohermaphroditism in male mice (1), confirming its involvement in sex differentiation rather than sex determination. Expression of Amh results from the interplay of several factors on the 360-bp Amh minimal promoter, including Sox9, the Wilms tumor-associated protein WT1, the GATA-binding protein GATA4, the orphan nuclear receptor DAX1 (NR0B1) (2), and the steroidogenic orphan nuclear receptor SF1 (Ad4BP, NR5A1). SF1 is central to the expression of Amh and cooperatively interacts with WT1 (3), GATA4 (4), and the HMG domain of Sox9 (5). How or, indeed, whether all four factors interact together on the Amh promoter is still unknown.

A number of SOX transcription factors have been shown to play important roles during sex determination and differentiation. For example, the Y-encoded testis-determining factor SRY is believed to be involved in directing the differentiation of the gonadal supporting cell lineage into Sertoli cells and thus acts as the sex determination switch in eutherian mammals (for review see Ref. 6). Complementary results have also implicated Sox9 as a male-specific sex determinant, with deletion or disruption of human Sox9 often resulting in XY sex reversal (7, 8) and overexpression of Sox9 inducing female to male sex reversal in mice (9).

Recently we showed that Sox8, a gene closely related to Sox9, is expressed in the developing brain, branchial arches, limbs, and kidneys and sex-specifically expressed in the testes (10). Structural comparison of Sox8 and Sox9 revealed considerable conservation, particularly within the HMG domain and the C-terminal trans-activation domain. We now report that Sox8 and Sox9 have overlapping expression patterns during testis development in mice and that both proteins bind specifically to the SOX binding site in the Amh promoter and interact with SF1 to synergistically enhance the expression of Amh. Thus we conclude that Sox8 may act redundantly with Sox9 to activate Amh expression during testis development.

MATERIALS AND METHODS

In Situ Hybridization—Whole-mount in situ hybridization was carried out essentially as described previously (11). Three Sox8 riboprobes corresponding to nucleotides 156–1447, 2746–2855, and 3595–4424 (GenBank™ accession number AF191325) were used together for Sox8 whole-mount in situ hybridization, to optimize the signal. The Oct4 riboprobe was generated from a plasmid kindly donated by Prof. Peter Rathjen and corresponds to nucleotides 594–1055 (GenBank™ NM_013653). Probes for Amh and Sox9 were synthesized as described previously (12, 13). Section in situ hybridization was carried out on 12-μm, serial sections of fresh-frozen 15 dpc testes and probed as previously described (14). Fluorescent color development was performed in place of NBT/BCIP staining and was achieved using “Fast red” tablets (Roche Applied Science) according to the manufacturer’s instructions. Post in situ immunofluorescence involved washing the sections in 0.1 M Tris-HCl (pH 8.2), followed by three washes in phosphate buffered saline (PBS, 5 min each). The sections were blocked for 1 h in 1% heat-inactivated sheep serum (HISS) in PBS. Anti-SOX9 antibody (15), diluted 1/200, was incubated with the sections overnight at 4 °C in 1% HISS, 0.1% Triton X-100 in PBS. Sections were washed.
three times in PBS, blocked for 5 min at room temperature in 1% HSS in PBS, and probed with anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) for 30 min at room temperature in 1% HSS, 0.1% Triton X-100 in PBS. Finally, sections were washed as before in PBS, mounted in 50% glycerol in PBS, and photographed.

**Cell Culture and Transfection Assays—Trans-activation domain analysis of GAL fusion constructs were performed as previously described (10). Co-transfection experiments were performed in triplicate using COS7 cells, in 12-well tissue culture plates containing 500 ng of reporter vector (360-bp Amh promoter in pGL3-basic, see Ref. 16). Transfection controls included 500 ng of pGL3-control. Transfections were performed using FuGENE6 (Bio-Rad) according to the manufacturer's instructions. Full-length SF1, SOX9, and SOX2 expression vectors were subcloned by sub-cloning the open reading frames from RSV/Ad4BP (16), Sox9-pSG5G (17), and cSox2-pCMV/SV1 (18) into pcDNA3.1+ (Invitrogen). Equal amounts of SOX8 (10), SOX9, and SOX2 expression constructs (20 ng per well) with or without SF1 expression construct (0 or 50 ng per well) were transfected, with the total amount of CMV-containing vector standardized by the addition of different amounts of pcDNA3.1+ to each transfection (total amount of CMV promoter-containing vector was 70 ng per replicate). Luciferase assays were performed essentially as previously described (10) with the following alteration: 80 μl of the 100-μl cell lysate was added to 100-μl Luciferase assay reagent (Roche Applied Science).

The statistical analysis performed on transfection data include a two-way, paired analysis of variance followed by the Student-Newman-Keuls post hoc analysis with a 95% confidence score. These analyses were performed using StatView 5.0.1 (SAS Institute Inc.).

**Electrophoretic Mobility Shift Assay—EMSA analysis was performed using recombinant, bacterially expressed GST fusion proteins of full-length SF1, SOX2, SOX8, and truncated SOX9 (amino acids 1–440). Recombinant plasmids were constructed using the vector pGEX-KG (19) for SOX2, -8, and -9 and pGEX-4T-3 (Amersham Biosciences) for SF1.

Wild-type and mutated Amh promoter fragments were cloned by PCR into the pGEM-Teasy vector (Promega) using MSt-wt, MSt-R1, MSt-R2, and MSt-R3 (20) as templates and the following primers: AATGAATGATGATGATG (upstream for amplifying R1, R2, and R3 fragments, AMHprovR2 (AGT GGA TCC CCC GGG CTG) for amplifying the wild-type fragment, together with AMHprovR (GGA ATT CGG CCC GTA CTA ACA CAA AA). Fragments were cut out of the respective constructs with EcoRI and gel-purified. Oligonucleotide probes (S2) harboring two SOX2 binding sites had the following sequence: GGG ATC CCT TTT GTC CTC GCA ATG CAC AAT ATT G. Fragments as well as oligonucleotide probes were labeled using γ-32P-ATP and T4 polynucleotide kinase. Binding reactions were performed in a final volume of 30 μl in binding buffer (100 mM KCl, 1 mM MgCl2, 10 mM Tris, pH 7.5, 4% glycerol, 0.1% Triton X-100, 1 mg/ml bovine serum albumin, 1 μg of poly(dI-dC)/poly(dA-dT), 0.5 mM dithiothreitol). All reactions contained 100 ng of purified protein were preincubated for 10 min at room temperature followed by the addition of end-labeled probe. After 20-min incubation at room temperature, DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels (containing 2.5% glycerol) at 150 V for 2 h in 1× TBE buffer.

**GST Pull-down Assays—**Fragments of SOX8 were generated using PCR and cloned into the pcDNA1.1+ expression vector. 32P-Labeled proteins were generated in vitro using the T7/Tnt system (Promega). GST-SF1 fusion protein was purified from BL21 cells as described previously (21) and left attached to glutathione-agarose beads. Bound SF1 fusion protein was then incubated with different 32P-labeled peptides in NTEN buffer (20 mM Tris Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) at 4°C for 1 h. The beads were then washed four times with NTEN, and bound proteins were eluted with 6× Laemmli buffer, boiled, analyzed on a 15% SDS-PAGE gel, and visualized by autoradiography.

**RESULTS**

**Sox8 Expression in Developing mouse Testes—**We have previously shown expression of Sox8 in mouse fetal testes at 13.5-day post coitum (dpc) (10). To determine the timing of expression of Sox8 in the testis, we performed a detailed in situ hybridization analysis of gonads at different developmental stages spanning sex determination and differentiation (11.5–16 dpc). Sox8 transcripts were first detected in the center of the testis at 12 dpc (25 tail somites), before cord formation (Fig. 1A). The expression domain expanded to include the rostral and caudal poles by 12.5 dpc and continued in testis cords beyond 16 dpc, with the strongest expression observed around 13.5–15 dpc. Expression of Sox8 in the ovary was undetectable from 11 to 16 dpc (Fig. 1A).

We next compared the expression of Sox8 with that of Sox9 and Amh, two key genes involved in testis development, to shed light on the potential regulatory relationships between these genes. At each stage studied, left and right genital ridges or testes from the same fetus were assessed in parallel for Sox8 and Sox9 expression. Sox8 and Amh expression was compared in other samples in a similar fashion (Fig. 1B). As a control, left and right gonads from the same fetus were assessed for differences in expression of each gene, and equal levels were observed in each case (data not shown).

The up-regulation of Sox8 expression was first evident at 11.5 dpc (18 tail somites, Fig. 1B), with expression continuing in the testis cords at 13 dpc through to 16 dpc. Similar to Sox8, expression of Sox9 began in the center of the testis and expanded to both poles by 12 dpc, when the first transcripts of Sox8 were detected in the center of the testis. Thus, Sox9 expression precedes that of Sox8 during sex determination by some 12 h. Sox8 and Amh expression patterns showed considerable overlap, with Amh transcripts first detected at 12 dpc in a patch of cells that was spatially more restricted than that expressing Sox8. The expression of Amh began in the center of the testis and encompassed the poles within 24 h; between 12 and 12.5 dpc the expression domain of Sox8 was more extended than that of Amh (Fig. 1B), suggesting that expression of the former preceded that of the latter. These data, combined with the localization of transcripts of all three genes to the developing testis cords, are consistent with the regulation of Amh expression by Sox9 and Sox8 in vivo.

Previous studies have established that Sox8 expression is associated with developing testis cords in mice (10, 22). To further test the hypothesis that Amh expression may be regulated by Sox8 in addition to SOX9, it was necessary to determine whether Sox8 is expressed in the Sertoli or germ cell component of the testis cords, because both Sox9 and Amh are known to be expressed in developing Sertoli cells (13, 17, 23). We performed in situ hybridization on serial sections of 15 dpc testes using probes for Sox8, Sox9, Amh, or the germ cell marker Oct4. Characteristic Sertoli cell expression was clearly evident for Amh, because it was distinct from the expression pattern of Oct4 (Fig. 2A). This analysis showed that Sox9 and Sox8 are also expressed in Sertoli cells (Fig. 2A). To confirm the expression of Sox8, Sox9, and Amh in the same cells, we performed double-labeling experiments, detecting Sox8 or Amh transcripts by in situ hybridization and SOX9 protein by immunofluorescence (Fig. 2B). All three genes were detected in the same cell type, as evident from the co-localization of signal observed in each case (Fig. 2B).

**Binding of Sox8 to Amh Promoter Sequences—**Based on the overlapping expression of Sox8 and Amh in Sertoli cells, we wished to determine whether Sox8 was capable of binding the critical SOX binding site in the Amh promoter (20). We performed an electrophoretic mobility shift assay (EMSA) with wild-type and mutant Amh promoter fragments spanning both the established SOX and SF1 binding sites (Fig. 3A). As expected, SF1 bound to promoter fragments with a wild-type SF1 binding site but not to those with a mutant SF1 site or an oligonucleotide (S2) harboring SOX2 binding sites (Fig. 3B, lanes 1–5), and SOX3 bound to wild-type but not mutant SOX binding site fragments (Fig. 3B, lanes 11–14). In these experiments, Sox8 also bound specifically to probes containing the wild-type but not the mutated SOX site (Fig. 3B, lanes 6–9).
thus demonstrating sequence-specific DNA binding of SOX8 within the Amh minimal promoter. In contrast, the unrelated SOX2 protein was not able to bind to any of the Amh promoter fragments (Fig. 3B, lanes 16–19). All three SOX proteins bound to the SOX2 binding site oligonucleotide S2 (Fig. 3B, lanes 10, 15, and 20). SOX8 DNA binding in vitro was consistently weaker than that shown by SOX9.

Activation of the Amh Promoter by SOX8 and SF1—SOX9 has previously been shown to act synergistically with SF1 to activate transcription from the minimal Amh promoter in cultured cells (5). Considering the temporal and spatial expression of Sox8, and the sequence-specific DNA binding to the Amh promoter, we tested directly whether SOX8 is able to stimulate Amh promoter activity. To investigate potential cooperative trans-activation between SF1 and SOX proteins, we performed co-transfection experiments similar to those described by de Santa Barbara and co-workers studying SOX9 and SF1 (5). COS7 cells were transfected with an Amh promoter-reporter construct and co-transfected with expression constructs for SF1 and the SOX proteins SOX8, SOX9, or SOX2.

In agreement with previous studies (5), SOX9 and SF1 synergistically activated transcription from the Amh promoter 4-fold above the levels observed with the reporter vector alone (Fig. 4A). SOX8 was also able to cooperatively activate transcription in the presence of SF1 (Fig. 4A). The synergistic activation of the Amh promoter by SF1 in combination with either SOX8 or SOX9 was found to be statistically significant with a confidence value of 0.05. In over a dozen independent experiments, the activation levels achieved by SOX8 and SF1 were consistently lower than those achieved by SOX9 and SF1, although the difference was not always statistically significant (Fig. 4A and B and data not shown). The unrelated SOX protein SOX2 was found to have no effect on Amh expression in the presence or absence of SF1 (Fig. 4A).

Finally, we also assessed whether SOX8 and SOX9 are able to act synergistically on the Amh promoter. The level of trans-activation achieved in the presence of SOX8, SOX9, and SF1 (around 5-fold, Fig. 4B) was similar to that observed for SOX9 and SF1.
and SF1 (difference not statistically significant). The small increase in trans-activation in the presence of all three factors shows that SOX8 and SOX9 do not act synergistically.

Potency of the SOX8 Activation Domain—Activation of the Amh promoter in the presence of SOX8 and SF1 was consistently lower than that observed with SOX9 and SF1. To further investigate this observation, we compared directly the relative trans-activation domain potency of SOX8 and SOX9 (Fig. 4C). The entire coding regions C-terminal to the HMG domains of SOX8 and SOX9 were fused to the GAL4 DNA binding domain and used in trans-activation domain analyses, similar to those performed in earlier studies (10, 15). These regions contain a conserved trans-activation domain at the C terminus and a second non-conserved trans-activation region (10, 24). The C terminus of SOX9 consistently showed an 80-fold increase in trans-activation compared with GAL alone, whereas SOX8 enhanced expression ~10-fold (Fig. 4C). Both fusion proteins were expressed at similar levels as detected by Western analysis (data not shown). These data suggest that SOX8 has a weaker C-terminal trans-activation domain than SOX9.

SOX8 and SF1 Interact Directly via the HMG Domain—Target specificity of SOX proteins is thought to be mediated by direct interaction with cell type-specific partner proteins and cofactors (25, 26). To support the in vivo significance of the observed synergy between SOX8 and SF1, we next tested the ability of SOX8 and SF1 to interact directly. Radiolabeled full-length SOX8 and fragments representing the N terminus, HMG domain, and C terminus were produced and incubated with GST-SF1 fusion protein attached to glutathione-Sepharose resin. After several washes, only full-length SOX8 and the HMG domain fragment remained bound by GST-SF1 (Fig. 5). These data confirm that SOX8 can bind to SF1 in vitro and that this interaction is mediated by the HMG domain of SOX8. Interestingly, SOX2 was also found to bind SF1 (Fig. 5), as might be expected for an interaction mediated by the highly conserved HMG domain. This latter observation is unlikely to
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**Fig. 3.** SOX8 binding to the Amh promoter. A, schematic representation of the Amh promoter DNA probes (wt, R1, R2, and R3 (20)) used in the EMSA. The confirmed SOX and SF1 binding sites are highlighted in black. The newly identified SOX binding site is marked by a box. The co-ordinates of the ends of the fragments and of the binding sites are listed below in base pairs relative to the ATG start codon for the Amh open reading frame. Site-directed mutations in the Amh promoters are represented in boldface and are underlined, with wild-type sequences represented by an unbroken line. B, EMSA of wild-type and mutant Amh promoter fragments with GST fusion proteins of SF1 (left panel), SOX8 (second panel), SOX9 (third panel), or SOX2 (right panel). Arrows indicate SF1, SOX8, and SOX9, respectively, binding as monomer. Arrowheads indicate SOX8 and SOX9 dimers. S2, oligonucleotide harboring two SOX2 binding sites (see "Material and Methods").

SOX8, Sox9, and Amh all show similar spatiotemporal expression patterns in developing mouse gonads, with expression beginning in the center of the genital ridge and spreading to the poles within 12 h. This is the first evidence that the expression of all three genes begins at, and expands from, the center of the developing testis, and is congruent with recent evidence that Sox8 expression also occurs as a wave beginning in the center of the testis and moving to the anterior and then posterior pole (28). The similar spatial and temporal dynamics of expression of Sry, Sox8, Sox8, and Amh provides support for a link between these genes in the molecular pathway of testis development. Sox8 expression is up-regulated in Sertoli cells around 12 dpc, ~12 h after Sox9 and just before Amh. These observations are consistent with the possibility that Sox8, like Sox9, can contribute to the regulation of Amh transcription in vivo.

This hypothesis is further supported by our results relating to SOX8 DNA binding and transcriptional activation in vitro. Our EMSA experiments demonstrated that SOX8 and SOX9 were able to bind as a monomer and, surprisingly, also as a dimer to two adjacent SOX binding sites in the Amh promoter. This is the first example of a paired SOX binding site in the promoter of a gene involved in sex differentiation. The binding to these sites is specific to SOX8 and SOX9, because the unrelated SOX2 protein did not bind. Similarly, it has been shown that SOX10 is able to bind to DNA as a monomer and a dimer (29). All three proteins (SOX8, -9, and -10) belong to the group E of SOX proteins, which contain a conserved 40-amino acid domain adjacent and N-terminal to the HMG-box. For SOX10 it has been demonstrated that this domain is essential for cooperative binding to response elements (30). So far, paired SOX binding sites have been only identified in the regulatory re-

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have any in vivo relevance, because SOX2 is not co-expressed with SF1 in Sertoli cells of the developing testis and does not activate transcription from the Amh promoter (Fig. 4A). Taken together, our observations suggest that SOX8 and SF1 can bind to adjacent sites on the Amh promoter and form a complex that is able to activate transcription from the Amh promoter in Sertoli cells.

**DISCUSSION**

The involvement of SOX genes in sex determination and differentiation is well documented, with the founding member of the family, Sry, shown to be the critical sex determining gene on the mammalian Y chromosome (27), and heterozygous SOX9 mutations often resulting in XY sex reversal in humans (7, 8). One of the early steps in sex differentiation is the up-regulation of Amh expression in Sertoli cells, which results in regression of the female-specific Müllerian duct and marks the onset of the male-specific hormonal pathway. All of the regulatory elements required for Amh expression during sexual differentiation are located within a relatively small, 180-bp promoter fragment (22), making this promoter particularly amenable to studying gene regulation. At least five factors are known to be involved in regulating Amh expression during sex differentiation. SF1 is believed to play a central role and has been shown to synergistically interact with SOX9 (5), GATA4 (4), or WT1 (3) to activate transcription, whereas interaction with DAX1 (2, 3) results in repression of Amh expression. In this study we show that SOX8 is also able to synergize with SF1 to enhance expression of Amh.

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regions of a number of target genes involved in chondrogenesis, such as Col2a1 and Col11a2 (31, 32). Mutation of these enhancer sequences has different functional consequences depending on which of the two SOX binding sites is mutated (29). Mutation of the primary SOX binding site (H11001 to 148 bp) in the Amh promoter results in pseudohermaphroditism in transgenic mice, demonstrating that this site is essential for Amh expression in vivo (20). The functional significance of the newly identified, adjacent SOX binding site, and whether SOX8 and/or SOX9 bind in vivo, is still unknown.

Similar to SOX9, SOX8 acts synergistically with SF1 to activate transcription from the Amh promoter, although the levels are consistently lower than those achieved by SOX9 with SF1. The reduced efficiency of SOX8 to trans-activate transcription in the presence of SF1 is most likely due to a combination of its apparent lower DNA binding affinity to Amh promoter sequences and its less potent trans-activation domain, compared with SOX9. Our observations suggest that adjacent binding as well as physical interaction are important for the synergy between SOX8 and SF1 in transcriptional activation, as it is for SOX9 and SF1.

Sox8 may perform functions additional to regulating Amh during sex determination. For instance, the expression of Sox9 is activated either directly or indirectly by SRY, but Sox9 expression continues long after the cessation of Sry expression (17, 23), suggesting that another element is required to maintain Sox9 expression. Sequence and cell transfection analyses have revealed a SOX binding site in the Sox9 promoter that is essential for maximal activity in gonadal somatic cells (33). Our

FIG. 4. Trans-activation of the Amh promoter by SOX8 and SF1. A, co-transfection of SOX8, SOX9, SOX2, and SF1 expression constructs with an Amh promoter reporter construct. The lanes and expression constructs transfected in each sample are represented below with “+” indicating the addition and “−” the omission of the expression construct. The -fold activation was compared with the reporter construct alone, lane 1. Error bars show ± S.E., with asterisks indicating samples significantly different between the presence and absence of SOX proteins. B, co-transfection of SOX8, SOX9, and SF1 with an Amh promoter reporter construct. The lanes, expression constructs transfected, -fold activation, error bars, and asterisks are indicated as for part A. C, SOX8 and SOX9 trans-activation domain comparison. GAL4 fusion constructs are shown on the left. The numbers represent mouse SOX8 or SOX9 amino acids fused with the GAL4 DNA binding domain. The right panel shows luciferase activity produced by each construct, relative to pLuc = 1, with standard error calculated from three replicate transfections.

FIG. 5. SOX8 interacts directly with SF1. Full-length SOX8, SOX9, SOX2, or fragments of SOX8 protein (SOX8-N, SOX8-HMG, or SOX8-C, as shown in the lower diagram) were produced in vitro. Radiolabeled proteins were incubated with either GST-SF1 (A) or GST-only (B). Electrophoresis of radiolabeled input proteins confirmed that all were intact and produced at similar levels (data not shown). Full-length SOX8, SOX9, SOX2, and fragments of SOX8 encoding the HMG domain can be seen to bind to SF1, indicating that this interaction is mediated by the HMG domain.
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observations suggest SOX8 as a candidate for a factor required to maintain Sox9 expression during sex differentiation.

Shen and Ingraham (34) showed recently that SOX8, like SOX9, can bind to a specific site in the Sf1 promoter. However, SOX8 was found to bind with much less affinity than SOX9, and was not able to specifically induce transcription from this promoter in vitro. These results provide an intriguing counterpoint to our present observations relating to Amh regulation, and it thus appears that the two SOX proteins are not able to act interchangeably in all situations. SOX8 appears to be capable of performing the same or similar function as SOX9 in regulating Amh expression during sex differentiation, but the overall extent of functional overlap between the two proteins remains to be determined. Functional redundancy has been demonstrated or proposed for a number of SOX proteins (18, 35–37) and is considered to reflect evolution. These possibilities are representative of a factor required to

represent another cellular context where SOX8 could contribute to the control of Amh expression. These possibilities are currently under investigation.

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