View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by University of Queensland eSpace

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 30, Issue of July 25, pp. 28101–28108, 2003
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

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

Received for publication, April 17, 2003
Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.M304067200

Goslik Schepers, Megan Wilson, Dagmar Wilhelm, and Peter Koopman‡

From the Institute for Molecular Bioscience, The University of Queensland, Brisbane 4072, Australia

Sox8 is a member of the *Sox* family of developmental transcription factor genes and is closely related to *Sox9*, a key gene in the testis determination pathway in mammals. Like *Sox9*, *Sox8* is expressed in the developing mouse testis around the time of sex determination, suggesting that it might play a role in regulating the expression of testis-specific genes. An early step in male sex differentiation is the expression of anti-Müllerian hormone (AMH) in Sertoli cells. Expression of the *Amh* gene during sex differentiation requires the interaction of several transcription factors, including SF1, SOX9, GATA4, WT1, and DAX1. Here we show that SOX8 may also be involved in regulating the expression of *Amh*. Expression of *Sox8* begins just prior to that of *Amh* at 12 days post coitum (dpc) in mouse testes and continues beyond 16 dpc in Sertoli cells. *In vitro* assays showed that SOX8 binds specifically to SOX binding sites within the *Amh* minimal promoter and, like SOX9, acts synergistically with SF1 through direct protein-protein interaction to enhance *Amh* expression, albeit at lower levels compared with SOX9. SOX8 and SOX9 appear to have arisen from a common ancestral gene and may have retained some common functions during sexual development. Our data provide the first evidence that SOX8 may partially compensate for the reduced SOX9 activity in campomelic dysplasia and substitute for *Sox9* where *Sox9* is either not expressed or expressed too late to be involved in sex determination or regulation of *Amh* expression.

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[1] 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_013633). 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

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An Australian Professorial Research Fellow of the Australian Research Council. To whom correspondence should be addressed. Tel.: 61-7-3365-4491; Fax: 61-7-3365-4388; E-mail: p.koopman@imb.uq.edu.au.

[1] The abbreviations used are: AMH, anti-Müllerian hormone; dpc, days post coitum; PBS, phosphate-buffered saline; HISS, heat-inactivated sheep serum; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; HMG, high mobility group.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

three times in PBS, blocked for 5 min at room temperature in 1% HISS in PBS, and probed with anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) for 30 min at room temperature in 1% HISS, 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 Cytofectene (Bio-Rad) according to the manufacturer's instructions. Full-length SF1, SOX9, and SOX2 expression vectors were constructed by sub-cloning the open reading frames from RSV/Ad4BP (16), Sox9-pSG5 (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 MIS-wt, MIS-R1, MIS-R2, and MIS-R3 (20) as templates and the following primers: AMHpro-for (AAG CTT GAT ATC GAA TTC CTG) for amplifying R1, R2, and R3 fragments, AMHpro-for2 (AGT GGA TCC CCC GGG CTG) for amplifying the wild-type fragment, together with AMHpro-rev (GGA ATT CGC CCC CTA TCA ACA CCA AA). Fragments were cut out of the respective constructs with *Eco*RI and gel-purified. Oligonucleotide probes (S2) harboring two SOX2 binding sites had the following sequence: GGG ATC CCT TTG TGT CTG GCA ATG CAC AAT ATT G. Fragments as well as oligonucleotide probes were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were performed in a final volume of 30 μl in binding buffer (100 mM KCl, 1 mM $MgCl_2$, 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). Reaction mixtures containing 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 0.5× TBE buffer.

GST Pull-down Assays—Fragments of SOX8 were generated using PCR and cloned into the pcDNA3.1+ expression vector. ^{35}S-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 ^{35}S-labeled proteins 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. 1*A*). 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. 1*A*).

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. 1*B*). 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 *Sox9* expression was first evident at 11.5 dpc (18 tail somites, Fig. 1*B*), 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. 1*B*), 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. 2*A*). This analysis showed that *Sox9* and *Sox8* are also expressed in Sertoli cells (Fig. 2*A*). 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. 2*B*). All three genes were detected in the same cell type, as evident from the co-localization of signal observed in each case (Fig. 2*B*).

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. 3*A*). 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. 3*B*, *lanes 1–5*), and SOX9 bound to wild-type but not mutant SOX binding site fragments (Fig. 3*B*, *lanes 11–14*). In these experiments, SOX8 also bound specifically to probes containing the wild-type but not the mutated SOX site (Fig. 3*B*, *lanes 6–9*),

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

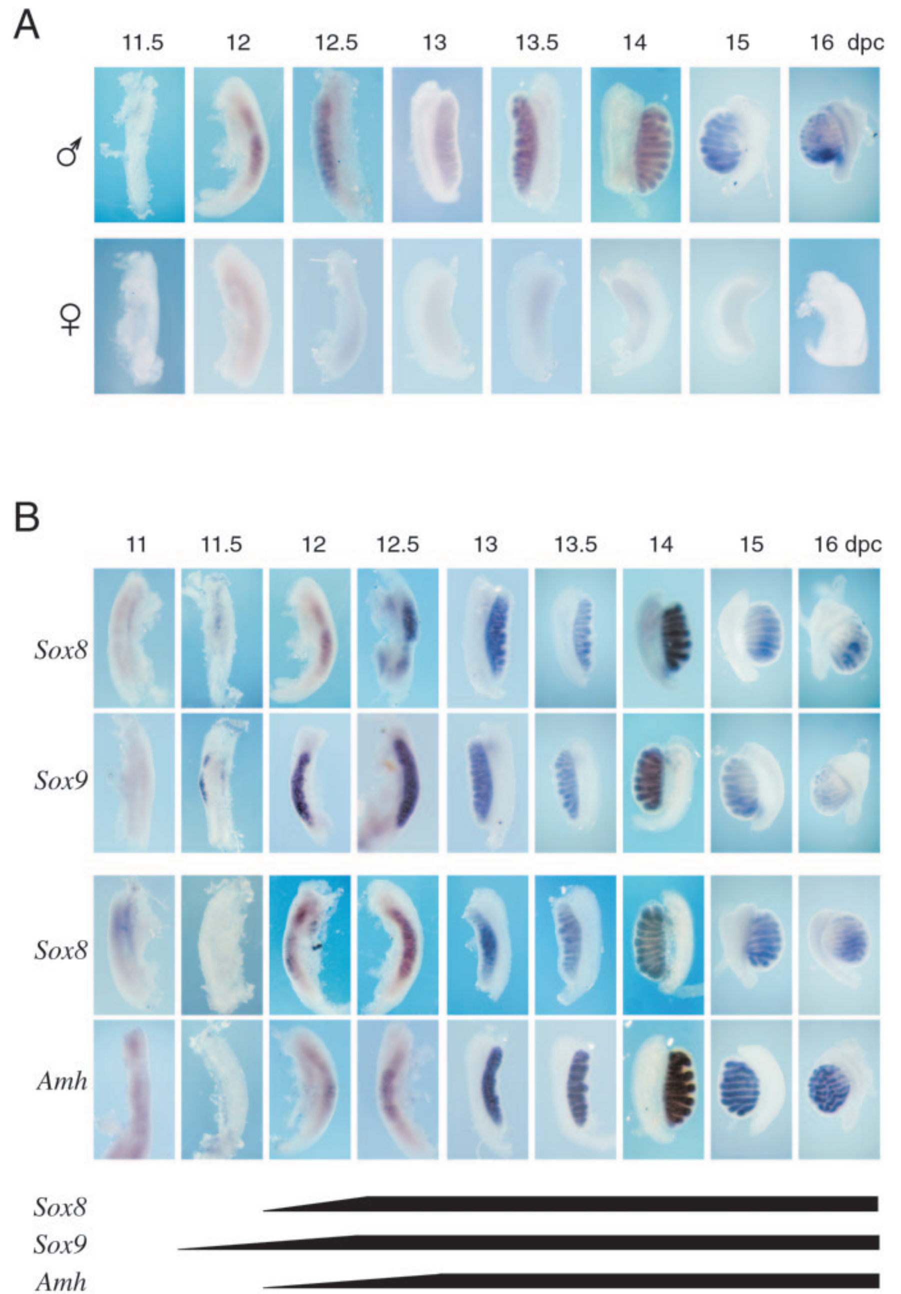

FIG. 1. **Expression of *Sox8*, *Sox9*, and *Amh* during sex determination.** *A*, whole-mount *in situ* hybridization of *Sox8* in developing testes (*male symbol*) and ovaries (*female symbol*). Developmental ages of gonads are labeled above. *B*, comparative expression of *Sox8*, *Sox9*, and *Amh*. Each column contains accurately staged gonads with the developmental ages labeled above. The *top two rows* represent pairs of left and right male gonads from the same embryos probed with *Sox8* (*first row*) or *Sox9* (*second row*). The *lower two rows* represent pairs of left and right male gonads from the same embryos probed with *Sox8* (*third row*) or *Amh* (*fourth row*). *Black lines* represent schematically the timing of expression of the three genes.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

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. 3*B*, *lanes 16–19*). All three SOX proteins bound to the SOX2 binding site oligonucleotide S2 (Fig. 3*B*, *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. 4*A*). SOX8 was also able to cooperatively activate transcription in the presence of SF1 (Fig. 4*A*). 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. 4 (*A* 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. 4*A*).

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. 4*B*) was similar to that observed for SOX9

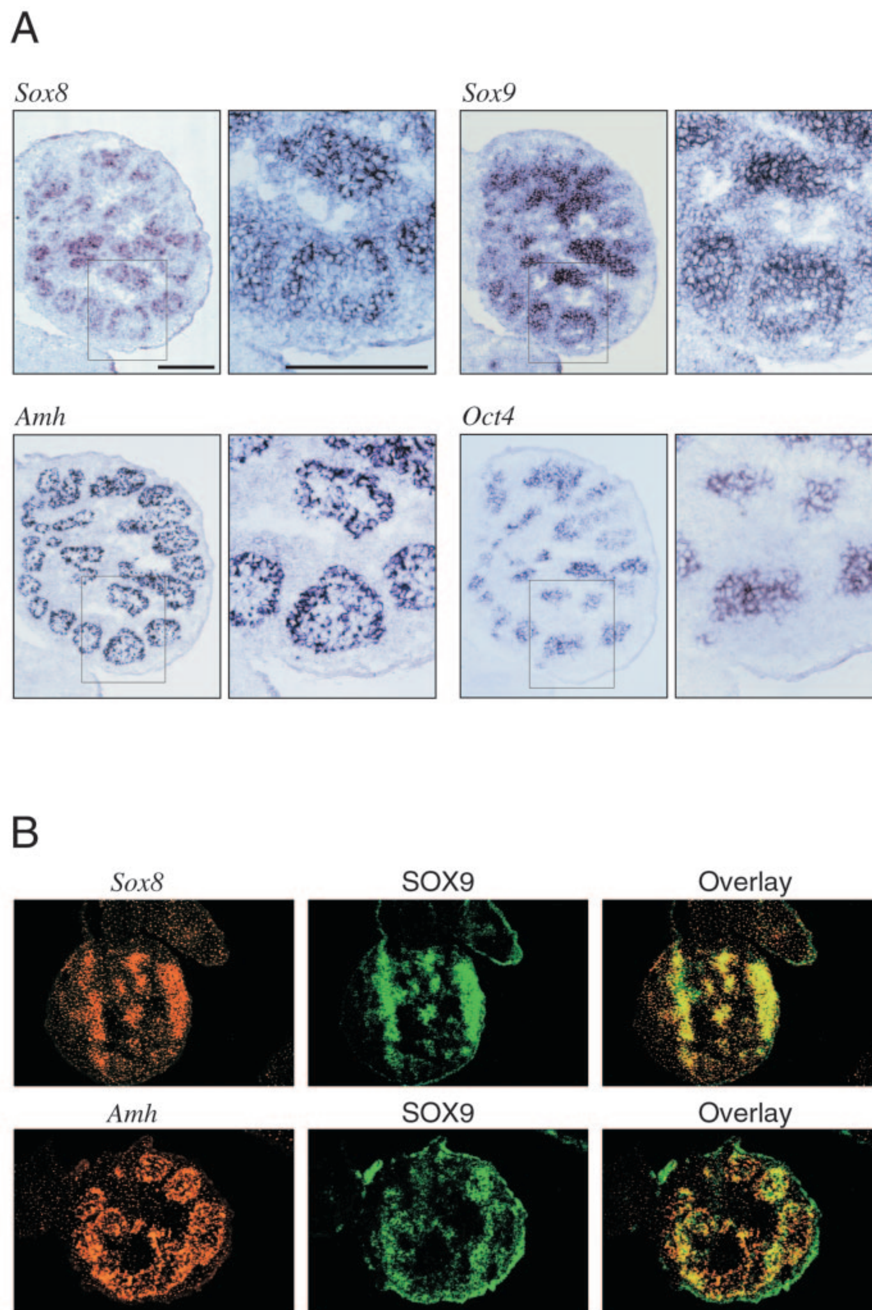

FIG. 2. **Sertoli cell expression of *Sox8*.** *A*, *in situ* hybridization of 15 dpc, serial, 12-μm cryosections probed with *Sox8* (*top row*, *left two panels*), *Sox9* (*top row*, *right two panels*), *Amh* (*lower row*, *left two panels*), and the germ cell marker, *Oct4* (*lower row*, *right two panels*). *Bar* = 0.1 mm. *Open boxes* represent regions enlarged in the neighboring *right panel*. *B*, co-labeling of *Sox8*, *Amh*, and SOX9. The *upper panel series* shows *Sox8 in situ* hybridization (*red*), SOX9 immunofluorescence (*green*), and an overlay with *yellow* representing overlapping signals. The *lower panel series* shows *Amh in situ* hybridization (*red*), SOX9 immunofluorescence (*green*), and an overlay with *yellow* representing overlapping signals.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

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. 4*C*). 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. 4*C*). 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

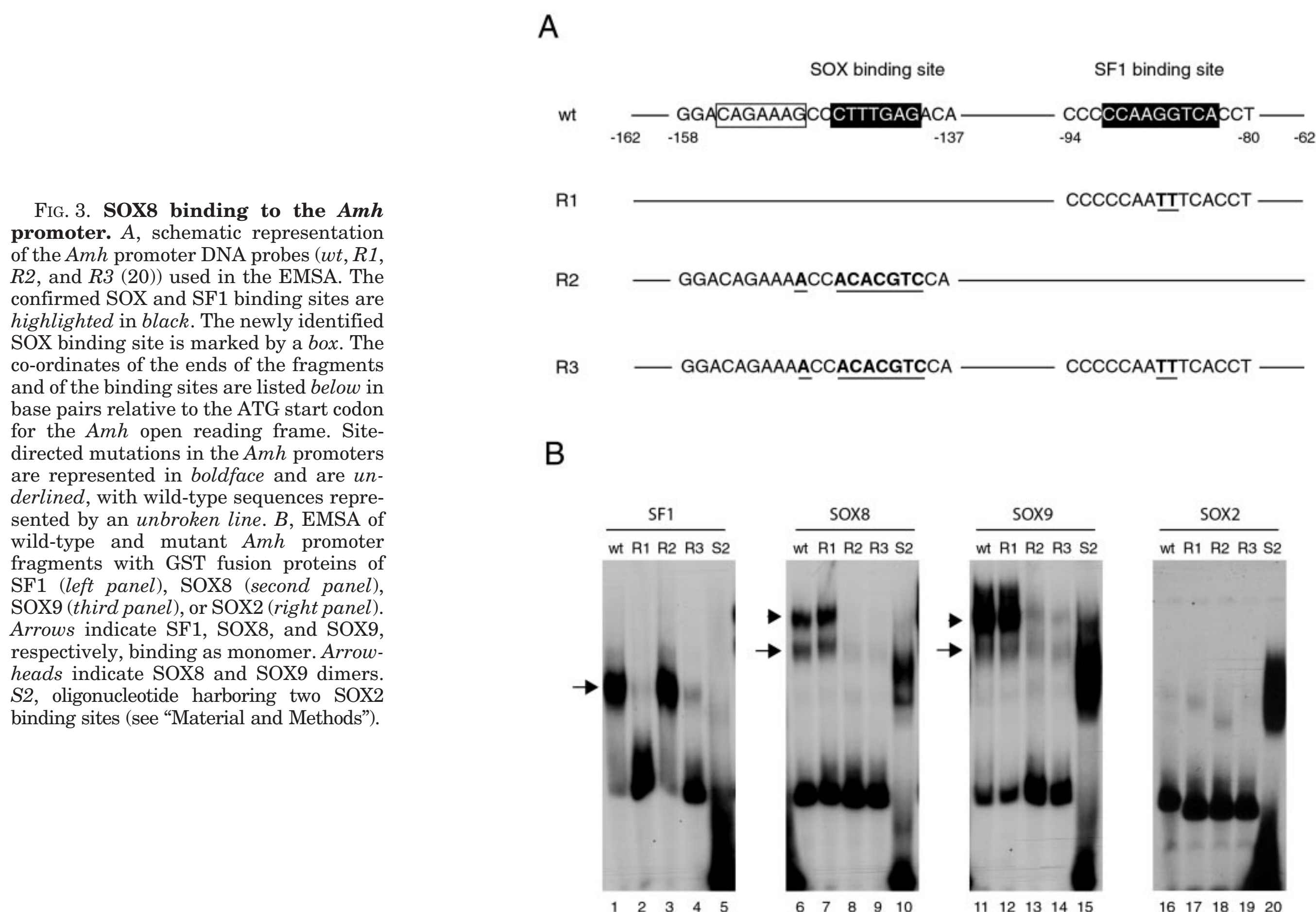

FIG. 3. **SOX8 binding to the *Amh* promoter.** *A*, schematic representation of the *Amh* promoter DNA probes (*wt*, *R1*, *R2*, and *R3* (20)) used in the EMSA. The confirmed SOX and SF1 binding sites are *highlighted* in *black*. The newly identified SOX binding site is marked by a *box*. The co-ordinates 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").

have any *in vivo* relevance, because SOX2 is not co-expressed with SF1 in Sertoli cells of the developing testis[2] and does not activate transcription from the *Amh* promoter (Fig. 4*A*). 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*.

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 *Sry* 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*, *Sox9*, *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-

[2] G. Schepers, M. Wilson, D. Wilhelm, and P. Koopman, unpublished observations.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

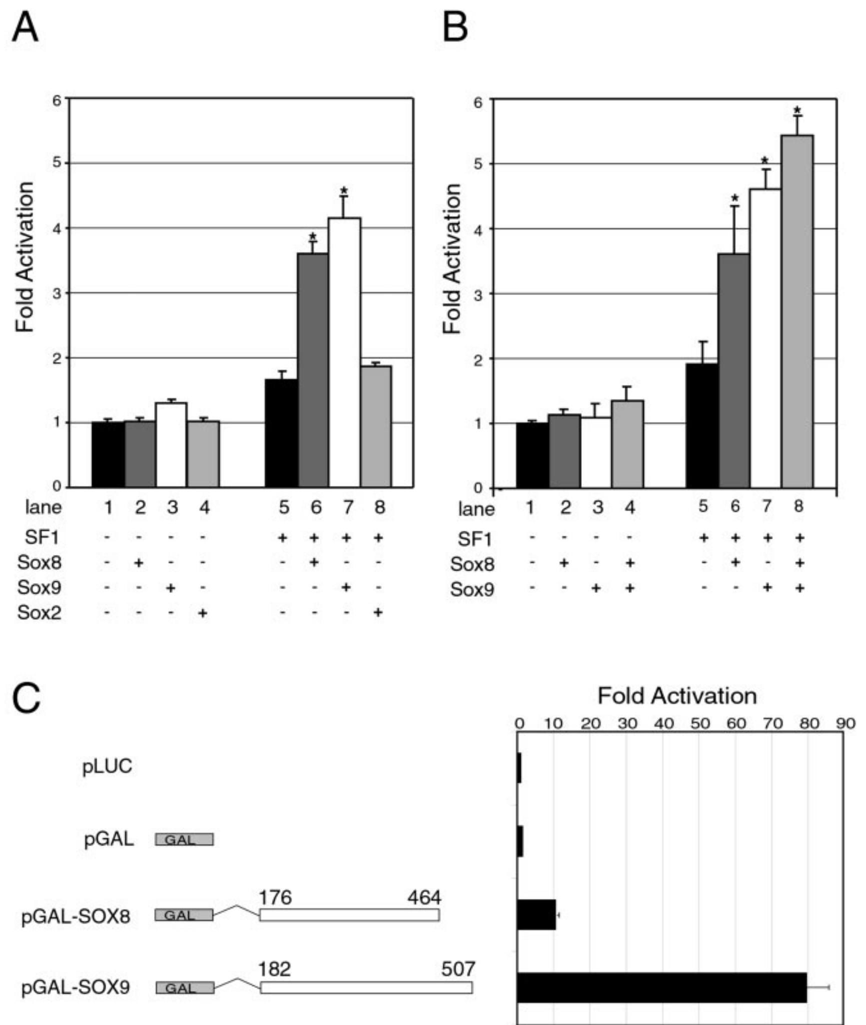

FIG. 4. ***Trans*-activation of the *Amh* promoter by SOX8 and SF1.** *A*, co-transfection of SOX8, SOX9, SOX2, and SF1 expression constructs with an *Amh* promoter reporter construct. The *lanes* and expression constructs transfected in each sample are represented below with "+" indicating the addition 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.

gions 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 (−148 to 154 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 binds *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 domains, 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

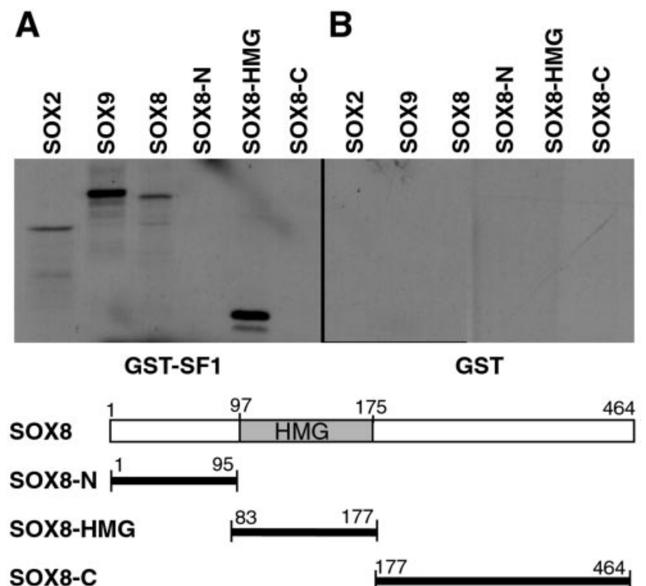

FIG. 5. **SOX8 interacts directly with SF1.** Full-length SOX8, SOX9, SOX2, or fragments of SOX8 protein (SOX8-N, SOX8-HMG, or SOX8-C, as shown in the *lower diagram*) were produced *in vitro*. Radiolabeled proteins were incubated with either GST-SF1 (*A*) or GST-only (*B*). Electrophoresis of radiolabeled 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.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

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 in progress. Often the genes involved are closely related and in the process of diverging, where their co-expression and shared functionality are a result of a recent common ancestor (38), as may be the case for *Sox8* and *Sox9* (39). They may also share common functions in other tissues where expression is overlapping, such as in the developing CNS, spinal cord, and kidneys (10, 12).

Sox8 and *Sox9* also show complementary expression patterns in numerous developing tissues, including the developing somites and skeleton. In the somites, *Sox9* expression is observed in the sclerotome (12) and *Sox8* in the dermomyotome (40). Similarly, *Sox9* is expressed in chrondrocytes (12) and *Sox8* in osteoblasts (41) during skeletal development. These complementary expression patterns may reflect the adoption of unique functions that were originally performed by a common ancestor. In teleosts, a lineage-specific, genome-wide duplication has resulted in two copies of many genes, and is often used as a model of gene duplication and divergence (38). Two *Sox9* genes have been identified in zebrafish, where the expression of the duplicate genes is distinct but overlapping in the brain, head skeleton, and fins (42). Considering these issues, we propose that the conserved function between SOX8 and SOX9 in regulating *Amh* expression may be an evolutionary remnant. The lower efficiency of SOX8 in regulating *Amh* expression suggests that this role may no longer be important *in vivo*, leaving SOX9 as the primary SOX protein required for this role, at least in eutherian mammals. This view is further confirmed with the apparent lack of sex reversal or gonadal dysgenesis observed in *Sox8* knockout mice (43).

In view of the biochemical similarity between SOX8 and SOX9, these proteins may share other roles during sexual development. This may explain the inconsistent rate of sex reversal seen in campomelic dysplasia patients (44), who have mutations in one copy of *SOX9*. We hypothesize that SOX8 at least partially ameliorates the haploinsufficiency of SOX9 in XY campomelic dysplasia patients who do not undergo sex reversal, with the penetrance of this effect likely to be influenced by other genetic modifiers.

It is clear that the SOX binding site in the *Amh* promoter is functionally important, because mutations in this site severely disrupt *Amh* expression levels *in vivo* (20). Previously it has been assumed that this site is important solely due to binding of SOX9. However, the ability of SOX8 to enhance expression of *Amh* in mammals through interaction with this site may explain the paradox in chicken and alligator sex determination, where *Amh* is expressed before *Sox9* (45, 46). In these species, SOX8 may be important for the regulation of *Amh* expression. *Amh* is also expressed in granulosa cells of the adult mammalian ovary in the absence of *Sox9* expression (17, 47). This may represent another cellular context where SOX8 could contribute to the control of *Amh* expression. These possibilities are currently under investigation.

Acknowledgments—We thank Murray Hargrave, Catherine Shang, and Rick Sturm for technical advice; Ken-Ichirou Morohashi for donating the RSV/Ad4BP clone and *Amh* reporter construct; Hisato Kondoh for donating the Sox2-pCMV/SV1 expression construct; Richard Behringer for donating the mutant *Amh* promoter constructs; Peter Rathjen for donating the *Oct4* probe; and Fred Martinson for help with statistical analysis. We are grateful to Kristy James, Kelly Loffler, and Josephine Bowles for comments on the manuscript and all the members of the Koopman laboratory for helpful discussions.

REFERENCES

1. Behringer, R. R., Finegold, M. J., and Cate, R. L. (1994) *Cell* **79,** 415–428
2. Tremblay, A., and Viger, R. (2001) *Biol. Reprod.* **64,** 1191–1199
3. Nachtigal, M. W., Hirokawa, Y., Enyeart-van Houten, D. L., Flanagan, J. N., Hammer, G. D., and Ingraham, H. A. (1998) *Cell* **93,** 445–454
4. Tremblay, J. J., and Viger, R. S. (1999) *Mol. Endocrinol.* **13,** 1388–1401
5. de Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Südbeck, P., Scherer, G., Poulat, F., and Berta, P. (1998) *Mol. Cell. Biol.* **18,** 6653–6665
6. Koopman, P. (1999) *Cell. Mol. Life Sci.* **55,** 839–856
7. Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, C., Weller, P. A., Weissenbach, J., Mansour, S., Young, I. D., Goodfellow, P. N., Brook, J. D., and Schafer, A. J. (1994) *Nature* **372,** 525–530
8. Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W., and Scherer, G. (1994) *Cell* **79,** 1111–1120
9. Vidal, V. P., Chaboissier, M. C., de Rooij, D. G., and Schedl, A. (2001) *Nat. Genet.* **28,** 216–217
10. Schepers, G. E., Bullejos, M., Hosking, B. M., and Koopman, P. (2000) *Nucleic Acids Res.* **28,** 1473–1480
11. Hargrave, M., and Koopman, P. (1999) in *In Situ Hybridisation Protocols* (Darby, I. A., ed) Vol. 123, 2nd. Ed., pp. 279–289, Humana Press, Totowa, NJ
12. Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., and Koopman, P. (1995) *Nat. Genet.* **9,** 15–20
13. Münsterberg, A., and Lovell-Badge, R. (1991) *Development* **113,** 613–624
14. Tsuchida, T., Ensini, M., Brenner-Morton, S., Baldassare, M., Edlund, T., Jessell, T. M., and Pfaff, S. L. (1994) *Cell* **79,** 957–970
15. Ng, L. J., Wheatley, S., Muscat, G. E. O., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P. L., Cheah, K. S. E., and Koopman, P. (1997) *Dev. Biol.* **183,** 108–121
16. Morohashi, K., Mukai, T., Kawabe, K., Suzuki, Mizusaki, H., Ishiharal, S., Umesono, K., and Kawajiri, K. (2000) *Endocrinol. Res.* **26,** 953
17. Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996) *Development* **122,** 2813–2822
18. Kamachi, Y., Cheah, K. S., and Kondoh, H. (1999) *Mol. Cell. Biol.* **19,** 107–120
19. Guan, K. L., and Dixon, J. E. (1991) *Anal. Biochem.* **192,** 262–267
20. Arango, N. A., Lovell-Badge, R., and Behringer, R. R. (1999) *Cell* **99,** 409–419
21. Hosking, B., Wyeth, J., Pennisi, D., Wang, S., Koopman, P., and Muscat, G. (2001) *Gene* (*Amst.*) **262,** 239–247
22. Shen, W.-H., Moore, C. C. D., Ikeda, Y., Parker, K. L., and Ingraham, H. (1994) *Cell* **77,** 651–661
23. Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., and Lovell-Badge, R. (1996) *Nat. Genet.* **14,** 62–68
24. McDowall, S., Argentaro, A., Ranganathan, S., Weller, P., Mertin, S., Mansour, S., Tolmie, J., and Harley, V. (1999) *J. Biol. Chem.* **274,** 24023–24030
25. Kamachi, Y., Uchikawa, M., and Kondoh, H. (2000) *Trends Genet.* **16,** 182–187
26. Wilson, M., and Koopman, P. (2002) *Curr. Opin. Genet. Dev.* **12,** 441–446
27. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991) *Nature* **351,** 117–121
28. Bullejos, M., and Koopman, P. (2001) *Dev. Dyn.* **221,** 201–205
29. Peirano, R., and Wegner, M. (2000) *Nucleic Acids Res.* **28,** 3047–3055
30. Schlierf, B., Ludwig, A., Klenovsek, K., and Wegner, M. (2002) *Nucleic Acids Res.* **30,** 5509–5516
31. Bell, D. M., Leung, K. K. H., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P. L., and Cheah, K. S. E. (1997) *Nat. Genet.* **16,** 174–178
32. Bridgewater, L. C., Lefebvre, V., and de Crombrugghe, B. (1998) *J. Biol. Chem.* **273,** 14998–15006
33. Kanai, Y., and Koopman, P. (1999) *Hum. Mol. Genet.* **8,** 691–696
34. Shen, J., and Ingraham, H. (2002) *Mol. Endocrinol.* **16,** 529–540
35. Nishiguchi, S., Wood, H., Kondoh, H., Lovell-Badge, R., and Episkopou, V. (1998) *Genes Dev.* **12,** 776–781
36. Lefebvre, V., Li, P., and de Crombrugghe, B. (1998) *EMBO J.* **17,** 5718–5733
37. Downes, M., and Koopman, P. (2001) *Trends Cardiovasc. Med.* **11,** 318–324
38. Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., and Postlethwait, J. (1999) *Genetics* **151,** 1531–1545
39. Bowles, J., Schepers, G., and Koopman, P. (2000) *Dev. Biol.* **227,** 239–255
40. Bell, K., Western, P., and Sinclair, A. (2000) *Mech. Dev.* **94,** 257–260
41. Harada, S., Grigoriadis, A. E., Nakayama, K., Oshima, H., Duong, L. T., and Rodan, G. A. (1999) *American Society for Bone and Mineral Research*, Vol. 14, pp. S144, 21st meeting vols., Blackwell Science, Inc., St. Louis, MO
42. Chiang, E. F., Pai, C. I., Wyatt, M., Yan, Y. L., Postlethwait, J., and Chung, B.

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

(2001) *Dev. Biol.* **231,** 149–163
43. Sock, E., Schmidt, K., Hermanns-Borgmeyer, I., Bosl, M. R., and Wegner, M. (2001) *Mol. Cell. Biol.* **21,** 6951–6959
44. Houston, C. S., Opitz, J. M., Spranger, J. W., Macpherson, R. I., Reed, M. H., Gilbert, E. F., Herrmann, J., and Schinzel, A. (1983) *Am. J. Med. Genet.* **15,** 3–28
45. Oreal, E., Pieau, C., Mattei, M. G., Josso, N., Picard, J. Y., Carré-Eusebe, D., and Magre, S. (1998) *Dev. Dyn.* **212,** 522–532
46. Western, P. S., Harry, J. L., Graves, J. A. M., and Sinclair, A. H. (1999) *Dev. Dyn.* **216,** 411–419
47. Ueno, S., Kuroda, T., Maclaughlin, D. T., Ragin, R. C., Manganaro, T. F., and Donahoe, P. K. (1989) *Endocrinology* **125,** 1060–1066

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016

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

Goslik Schepers, Megan Wilson, Dagmar Wilhelm and Peter Koopman

J. Biol. Chem. 2003, 278:28101-28108.
doi: 10.1074/jbc.M304067200 originally published online May 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304067200

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 15 of which can be accessed free at
http://www.jbc.org/content/278/30/28101.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at UQ Library on October 16, 2016