The Transcription Factors Steroidogenic Factor-1 and SOX9 Regulate Expression of Vanin-1 during Mouse Testis Development*

Received for publication, November 12, 2004
Published, JBC Papers in Press, December 8, 2004, DOI 10.1074/jbc.M412806200

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We previously showed, using differential expression screening and in situ hybridization that Vanin-1, which encodes a glycosylphosphatidylinositol-linked membrane-associated pantetheinase, is expressed in a sex-specific manner during fetal gonad development in mice (Bowles, J., Bullejos, M., and Koopman, P. (2000) Genesis 27, 124–135). In the present study we investigate in detail the expression and regulation of Vanin-1 in the fetal testis. Vanin-1 is co-expressed with the transcription factors steroidogenic factor-1 (SF-1) and SOX9 in Sertoli cells and, at a lower level, with SF-1 in Leydig cells in developing testes. SF-1 is able to activate the transcription of the Vanin-1 promoter in in vitro reporter assays, and this activation is further augmented by SOX9. We found that SF-1 is able to bind to two sites in the Vanin-1 promoter, whereas SOX9 can bind to a single interposed site defined by DNA footprinting. Mutation of the SF-1 or SOX9 sites disrupts the binding of these factors and activation of transcription. The expression of Vanin-1 was abolished in Leydig cells of a mouse mutant lacking SF-1 in that cell type. Our findings account for the sex- and cell-type-specific expression of Vanin-1 in the developing mouse gonad in vivo, which we suggest is required to provide an appropriate environment for male germ cell development.

Male and female gonads, although structurally and functionally quite distinct tissues, arise in the embryo from the same tissue primordia, the genital ridges. Their developmental trajectories begin to diverge about 10.5 days post coitum (dpc) in mice, when Sry expression in XY genital ridges initiates testis determination. Many genes that operate downstream of Sry have been identified as being expressed male-specifically during sex determination and gonad differentiation, but little is known about how they are regulated, interact, and function within the overall scheme of sex differentiation.

SRY is the eponymous founding member of the Sox (SRY-related HMG box) gene family, encoding a group of proteins that bind to DNA in a sequence-specific manner via their HMG (high mobility group) domain (1). Immediately following Sry induction the expression of SOX9, another member of the SOX family, is up-regulated in male genital ridges. Transgenic mouse studies have shown that expression of SOX9 is sufficient for testis formation (2), whereas mutations in human SOX9 can result in XY sex reversal (3). These observations show that SOX9 is a key transcription factor in the male sex determination pathway.

SOX9 plays an essential role in regulating the gene encoding anti-Müllerian hormone (AMH), a key hormone required for the regression of the female duct system in male embryos. A homozygous mutation of the SOX9 binding site within this promoter in male mice ablates the expression of Amh, resulting in the retention of the Müllerian ducts and the generation of pseudohermaphrodites (4).

In conjunction with SOX9, steroidogenic factor 1 (SF-1) is also required to modulate levels of Amh expression (4, 5). SF-1 (also known as Ad4BP or NR5A1) is an orphan nuclear receptor required for gonad and adrenal gland development that is expressed in the Leydig and Sertoli cells of the testis and in the adrenal cortex (6, 7). Mice homozygous for a null mutation of SF-1 fail to develop adrenal glands and gonads, establishing SF-1 as an essential regulator required for their development (8). Many potential target genes for SF-1 have been determined, including several genes involved in steroidogenesis in the adrenal gland and Leydig cell lineage of the testis. Two of these targets encode proteins required for the rate-limiting steps in testosterone synthesis: steroidogenic acute regulatory (STAR) protein, which regulates cholesterol uptake into the mitochondria, and cholesterol side chain cleavage enzyme, which catalyzes the first cleavage reaction in steroid biosynthesis (9).

In vitro studies suggest that the HMG domain of SOX9 interacts directly with the C-terminal domain of SF-1 to cooperatively activate expression from the Amh promoter in Sertoli cells (10). Recently, we demonstrated that a related SOX factor expressed by Sertoli cells, SOX8, can synergize with SF-1 in a similar fashion (11). There is further evidence to suggest that SOX9 may be responsible for the male-specific expression of SF-1, as it can bind to the proximal promoter of SF-1 and up-regulate expression in vitro (12). Although a large body of data indicates that SF-1 and SOX9 are critical transcription factors required for embryonic testis differentiation and are likely to cooperate in several steps of this process, only Amh has been identified as a direct target for these proteins.

Vanin-1 has been identified as being expressed in a male-enriched fashion in the embryonic gonad by both subtractive hybridization (13) and microarray screening (14). Whole mount...
in situ hybridization of XY embryonic gonads has shown that Vanin-1 is expressed immediately following Sry expression, just before the formation of the testis cords. Vanin-1 is a member of a protein family consisting of at least three members in humans (Vanin-1, -2, and -3) and two members in mice (Vanin-1 and -3) (15). The Vanin-1 gene product is a glycosylphosphatidylinositol-linked membrane-associated pantothenase, a family of enzymes that catalyzes the hydrolysis of pantotheine into pantothenic acid (vitamin B5) and cysteamine, an anti-oxidant (16, 17). Vanin-1 null mice develop normally, but tissues that would typically express this protein lack cysteamine and exhibit a modified stress response (17–19). Recently, it has been demonstrated that expression from the Vanin-1 proximal promoter is up-regulated in response to oxidative stress (19), further suggesting that Vanin-1 is likely to play an important role in tissue response to stress and inflammation.

To identify proteins involved in regulating expression from the Vanin-1 promoter in the developing testis, immunofluorescence studies were carried out on cryosectioned embryonic testes. Vanin-1 is expressed by cells that are positive for SF-1 and is strongly expressed in Sertoli cells that express both SOX9 and SF-1. The proximal Vanin-1 promoter was isolated and found to contain putative binding sites for both SF-1 and SOX9. Electroreflective mobility shift assays and cell transfection assays showed that SF-1 and SOX9 synergistically activate transcription from this promoter.

EXPERIMENTAL PROCEDURES

PCR and Plasmid Constructions—The oligonucleotide primers 5'-GATTCCTGTGATCAACCTC-3' and 5'-CATGCTGAATCTCAAAAGA-3' were used to amplify the Vanin-1 promoter fragment (267 bp) from mouse genomic DNA by PCR. The resulting fragment was cloned into pGEM-T Easy (Promega), sequenced, and then subcloned into pGL2-Basic (Promega) using the KpnI and SacI restriction sites. Expression constructs for SOX9, SOX8, and SF-1 and GST fusion constructs were reported previously (11). The open reading frame of Sry was amplified by PCR using the oligonucleotide pair 5'-GCGGGATCCATGGGAGG-GCCATGTCAG-3' and 5'-GCGCTGACCTGATGACTGCCCACAC-CA-3' and cloned into pGEM-T Easy (Promega), sequenced, and then subcloned into pGL2-Basic (Promega) using the KpnI and SacI restriction sites. Expression constructs for SF-1 and SOX9 were made using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The sequence of the top strand oligonucleotide used to generate each mutation was as follows, with the nucleotides mutated in bold, SF-M1 5'-CTGACCAGAAATTAGCTGATATCTCGAAACCTGTGTTG-3', SF-M2 5'-TTTTTTGAGGCTACCCAGTTGCACGGGAAACACGTG-3', and MutSOX 5'-GCTAATGAAACCTCATTGCCTGTGACTTGTTG-3'. The presence of introduced mutations was confirmed by DNA sequencing.

Electrophoretic Mobility Shift Assay—EMSA analysis was performed as described previously (11). Briefly, KpnI-SacI restriction fragments from pGL2-Vnn expression constructs were purified using the Qiagen Gel Purification Kit. Following dephosphorylation using alkaline phosphatase, fragments were end-labeled with 32P ATP and 74 nucleotides kinase. Probes were purified on a Nick Purification Column (Amersham Biosciences) and eluted in TE buffer (50 mM Tris-Cl, 1 mM EDTA, pH 8).

DNase I Footprinting—The procedure for DNase I footprinting has been described previously (22). The Vanin-1 promoter fragment was labeled on either strand by PCR by using one 32P end-labeled oligonucleotide primer (for the strand to be labeled) along with the non-labeled primer. Footprinting reactions were electrophoresed in parallel with sequencing reactions to determine the sequence of the protected site. Sequencing reactions were performed using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Biosciences) using the oligonucleotide that was labeled in the PCR.

Luciferase Assays—TM3 cells (23) were plated in 12-well plates and transfected with 500 ng of luciferase reporter plasmid with either empty expression vector (pcDNA3) or SF-1 (50 or 20 ng) and/or SOX9 (20 ng) expression plasmids using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed twice with PBS and assayed for luciferase activity with the Luciferase Reporter Gene assay kit (Roche Applied Science). Each transfection was done in duplicate and repeated independently at least three times; data represent the average ±fold increase relative to cells co-transfected with empty expression vector (pcDNA3).

Immunofluorescence—Antibody staining was performed on cryosectioned embryonic tissue with the following antibodies. Vanin-1 antibody (1:100 dilution) was purified from the rat hybridoma clone H202-407-6-3 as described previously (24). Rabbit anti-SF-1 antibody (1:1000 dilution) was a kind gift from K. Morohoshi (National Institute for Basic Biology, Okazaki, Japan). Goat anti-AMH antibody (sc-6886) was purchased from Santa Cruz Biotechnology and used as a 1:100 dilution. Rabbit anti-SOX9 antibody has been described previously (25). Secondary antibodies (anti-rat Alexa 488, anti-rabbit Alexa 594, and anti-goat Alexa 568) were purchased from Molecular Probes and used at a 1:200 dilution.

Tissue samples were fixed with 4% paraformaldehyde in PBS for 1 h, washed with PBS, and then incubated overnight in PBS containing 30% sucrose. Samples were then frozen in OCT cryo-embedding compound and cut into 10-μm sections at −20°C. Slides were washed three times with PBS (containing 0.1% Triton X-100) and blocked with 10% heat-inactivated horse serum. Slides were incubated overnight at 4°C with the primary antibody diluted in blocking solution as indicated above. After three washes with PBS, slides were incubated with the appropriate secondary antibody, washed, mounted, and imaged using a Bio-Rad Radiance 2010 MP confocal microscope.

Gill mid-specific SF-1 null mice were bred and genotyped as described previously (26). Embryos were isolated and prepared as described above for sectioning.

RESULTS

SF-1 and SOX9 Are Co-expressed with Vanin-1 in Vivo—Previously it has been shown by in situ hybridization on whole gonads that Vanin-1 mRNA is expressed predominantly in Sertoli cells of the testis cords of mouse XY fetal gonads at 13.5 dpc (13, 14). In the present study, immunofluorescence was carried out to further characterize the expression of Vanin-1 in the embryonic testis in terms of cell type and possible co-expression with transcription factors present in the Sertoli cells, SOX9 and SF-1 in vivo. Mouse fetal testes at 13.5 and 16.5 dpc were sectioned and stained with an antibody specific for Vanin-1 (24), together with anti-SF-1, -SOX9, or -AMH antibodies (Fig. 1). Vanin-1 was found to be strongly expressed in the cytoplasm of Sertoli cells at these stages, as evidenced by colocalization with AMH (Fig. 1A) and SOX9 (Fig. 1C). Some staining was also observed in interstitial cells located between the cords. These interstitial cells were identified as Leydig cells by positive staining for SF-1 (6) (Fig. 1B). These results showed that Vanin-1 protein is expressed in Leydig cells as well as Sertoli cells.

SF-1 is known to be expressed strongly in the adrenal cortex at 13.5 dpc (7). Vanin-1 staining was found on the surface of SF-1-positive cells within the adrenal cortex (Fig. 2). Antibody staining was weaker compared with the Vanin-1 staining found in the testis cords (Fig. 2). Together these results indicate that cells positive for SF-1 also express Vanin-1 and that there may be stronger expression of Vanin-1 in cells that also express SOX9 in vivo, consistent with the possibility that SOX9 and SF-1 may together be involved in the regulation of Vanin-1 transcription.

SF-1 and SOX9 Can Bind to the Vanin-1 Proximal Promoter—To investigate whether SF-1 and SOX9 can activate transcription from the proximal promoter of Vanin-1, a 267-bp fragment of the promoter was cloned by PCR using mouse genomic DNA. A sequence analysis revealed two potential SF-1 binding sites based on the consensus binding sequence previously determined for SF-1 (27) (Fig. 3A). To test whether SF-1 is able to bind to this promoter fragment, we performed EMSA using a
SF-1 and SOX9 Activate the Vanin-1 Promoter

Vainin-1 expression is stronger in the testis cords where SOX9 is expressed compared with Leydig cells or the adrenal where Vanin-1 expression is stronger in the testis cords where SOX9 is expressed (Fig. 2), the ability of SOX9 to bind to the Vainin-1 promoter was investigated. Purified GST-SOX9 fusion protein was able to bind to the isolated promoter fragment in EMSA (Fig. 3C) confirming the presence of a SOX site(s) within the promoter.

In addition to SOX9, Sertoli cells also express SRY (28) and SOX8, a protein that shares 53% identity with SOX9 (11). As with SOX9, SOX8 has also been shown to cooperate with SF-1 to activate transcription from the Amh promoter (11), although SOR8 is not crucial for testis formation because Sox8 null mice develop normally (29). Analysis of Sox8/Sox8 double mutant mice revealed that Sox8 complements SOX9 function during testis differentiation (30). Because all SOX proteins, including SRY, share the same consensus binding sequence (31), we next tested whether SRY and SOX8 could bind to the Vainin-1 promoter fragment. Interestingly, GST-SOX9 fusion protein was able to bind to the isolated promoter fragment (Fig. 3C) confirming the presence of a SOX site(s) within the promoter.

To locate the putative SOX factor binding site(s) within the Vainin-1 proximal promoter fragment, DNase I footprinting analysis was carried out (Fig. 4A). The Vainin-1 promoter was labeled on one strand and incubated with either GST or GST-SOX9 fusion protein. The inclusion of purified GST-SOX9 protein resulted in a protection of bases −206 to −173 (Fig. 4A) from DNase I digestion. This protected region of 34 bases is of a similar size to that protected in a footprinting analysis carried out on the Amh promoter (10). To confirm this was the

GST-SF-1 fusion protein. The Vainin-1 promoter probe formed DNA-protein complexes with GST-SF-1, but not with GST alone (Fig. 3B, arrowheads), confirming the presence of SF-1 binding sites within this promoter fragment. To test for binding specificity, each site was mutated and tested by EMSA for the ability to bind GST-SF-1. A mutation of either site individually produced a minor reduction of SF-1-DNA complex formation, but when both sites were mutated in the same fragment the formation of a specific SF-1-DNA complex was abolished (Fig. 3B).

Because SF-1 and SOX9 cooperatively act to up-regulate Amh promoter expression, and in view of our observations that
SF-1 and SOX9 Activate the Vanin-1 Promoter

To determine whether the sites for SF-1 and SOX9 that we identified are functional in regulating Vanin-1 expression, the proximal promoter was cloned immediately upstream of the luciferase gene in the pGL2-Basic reporter construct (pGL2-Vnn). TM3 cells, derived from mouse fetal gonads (23) and retaining expression of a number of genes that SOX9 required the presence of SF-1 to activate transcription, indicating that it cannot substitute for SOX9 in vitro (10) even though it can bind directly to SF-1.2

SOX transcription factors bind to DNA via their HMG domains, resulting in bending of the DNA by up to 71°, and has been proposed to act as an architectural transcription factor (33, 34). To determine whether SOX9 synergizes with SF-1 to activate the Vanin-1 promoter through DNA bending or in a more conventional manner through its trans-activation domain, TM3 cells were co-transfected with an expression construct, pcDNA3-SOX9/TA, encoding a SOX9 mutant protein that lacks the C-terminal trans-activation domain but can still bind to DNA. In luciferase assays this construct failed to activate transcription from the Vanin-1 promoter over levels seen with SF-1 alone (Fig. 5A). Thus promoter regulation depended on the SOX9 trans-activation domain.

To confirm that the binding site(s) responsible for activation by SF-1 and SOX9 had been identified, reporter constructs containing mutations in SF-1 site 1 (SF-M1), site 2 (SF-M2), or both (SF-M1M2), or in the SOX site (MutSox) were co-transfected with pcDNA3-SF-1. Mutation of either SF-1 site reduced SF-1-dependent activation from the Vanin-1 promoter to ~28% of wild-type promoter expression levels. Mutation of both SF-1 sites completely abolished promoter activity (Fig. 5B). This indicates that both binding sites are required for transcriptional activation by SF-1. Previous analysis of the Amh promoter, which also contains two SF-1 DNA binding sites, found that mutation of either site greatly reduced promoter activity demonstrating also that both sites were required for full promoter activation by SF-1 (35).

Co-transfection of pcDNA3-SOX9 and pcDNA3-SF-1 with the Vnn1 promoter-reporter construct containing the mutated SOX site (MutSox) identified by DNase I footprinting produced luciferase levels similar to pcDNA3-SF-1 transfection alone (Fig. 5B). Thus the putative SOX binding site identified in this study is essential for activation of transcription from the Vanin-1 promoter by SOX9.

Disruption of SF-1 Function Leads to Loss of Expression of Vanin-1 in Vivo—Recently, targeted excision of the SF-1 gene in fetal Leydig cells in vivo has been reported, mediated by Cre-recombinase expression under the control of the AmhR2 promoter (26). The developing SF-1-null testes are smaller in size, show delayed cord formation, and lack expression of cholesterol side chain cleavage enzyme and STAR, indicating that these genes are in vivo targets of SF-1. We analyzed 14.5-dpc testes from knock-out and wild-type littermates by immunofluorescence with the Vanin-1 antibody, to test for a role of SF-1 in expression of Vanin-1 in vivo. Mutant gonads showed no interstitial (Leydig) cell expression of Vanin-1, but expression persisted in Sertoli cells, which retain the expression of SF-1 in these mice (26), as evidenced by co-staining with antibodies against the Sertoli cell marker AMH (Fig. 6). In wild-type littermates, Vanin-1 was co-expressed with AMH in the Sertoli cells and also expressed in the Leydig cells (Fig. 6). This indicates that removing SF-1 expression in Leydig cells prevents expression of Vanin-1 in these cells and that Vanin-1 is a target gene of SF-1 in vivo.

Fig. 4. Identification of the SOX binding site in the Vanin-1 promoter. A, DNase I footprinting analysis. The non-coding strand of the same double-stranded DNA fragment used the EMSA studies above was labeled with 32P and incubated with 100 ng of purified GST only or GST/SOX9 protein. Following DNase I digestion, the fragments were electrophoresed alongside each other along with sequencing reactions for the same DNA. A solid box indicates the GST/SOX9 protected region. B, mutations in a possible SOX site were introduced into the promoter by site-directed mutagenesis. Purified GST/SOX9 was incubated with 32P-labeled wild-type or mutated (MutSox) Vanin-1 DNA. GST/SOX9 failed to form a complex with the mutated DNA fragment.

SOX site bound by SOX9, we next used site-directed mutagenesis to induce mutations into this putative SOX binding site and tested by EMSA. A mutation of this site prevented both SOX9 and SOX8 from binding to the promoter fragment (Fig. 4B), suggesting that the correct binding site for these proteins had been identified.

SF-1 and SOX9 Co-activate Transcription from the Vanin-1 Proximal Promoter—To determine whether the sites for SF-1 and SOX9 that we identified are functional in regulating Vanin-1 expression, the proximal promoter was cloned immediately upstream of the luciferase gene in the pGL2-Basic reporter construct (pGL2-Vnn). TM3 cells, derived from mouse fetal gonads (23) and retaining expression of a number of genes of the sex-determining pathway (32), were co-transfected with pGL2-Vnn without or with an SF-1 expression plasmid (pcDNA3-SF-1) and were assayed for luciferase activity after 48 h. Co-transfection with 20 ng of pcDNA3-SF-1 resulted in a 22-fold increase in luciferase activity compared with the empty pcDNA3 vector (Fig. 5A). This response was dose-dependent, as a transfection of 50 ng further increased the promoter activity 35-fold above basal activity.

To investigate possible synergy between SF-1 and SOX9 in activating Vanin-1 transcription, TM3 cells were co-transfected with the pGL2-Vnn reporter plasmid without or together with pcDNA3-SOX9 and/or pcDNA3-SF-1 expression plasmids. No activation was found in cells transfected with pcDNA3-SOX9 alone (Fig. 5A). However, co-transfection of pcDNA3-SOX9 and pcDNA3-SF-1 resulted in a doubling in the relative levels of luciferase produced compared with pcDNA3-SF-1 alone (Fig. 5A). These results demonstrated that SOX9 required the presence of SF-1 to activate transcription and that it can act synergistically with SF-1 to mediate expression from the Vanin-1 promoter.

Our EMSA experiments showed that also SOX8, but not SRY, is able to bind the Vanin-1 promoter. Expression constructs pcDNA3-SOX8 and pcDNA3-SRY were assayed to determine whether these SOX factors could stimulate activity from the Vanin-1 promoter in reporter assays. Cells transfected with pcDNA3-SOX8 showed an increase in luciferase activity compared with cells transfected with pcDNA3-SF-1 alone (Fig. 5A). This suggests that SOX8 can, like SOX9, bind to and activate transcription from the Vanin-1 promoter in combination with SF-1. pcDNA3-SRY failed to activate the transcription of pGL2-Vnn either alone or in combination with pcDNA3-SF-1. This situation is similar to that reported for the Amh promoter where SRY was also unable to activate transcription, indicating that it cannot substitute for SOX9 in vitro (10) even though it can bind directly to SF-1.2

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Co-transfection of pcDNA3-SOX9 and pcDNA3-SF-1 with the Vnn1 promoter-reporter construct containing the mutated SOX site (MutSox) identified by DNase I footprinting produced luciferase levels similar to pcDNA3-SF-1 transfection alone (Fig. 5B). Thus the putative SOX binding site identified in this study is essential for activation of transcription from the Vanin-1 promoter by SOX9.
DISCUSSION

Previous studies have shown that SOX9/SOX8 and SF-1 interact to activate transcription from the Amh promoter (10, 11). Here we demonstrate that the promoter of the Vanin-1 gene also can be activated cooperatively by SOX9/SOX8 and SF-1. We found that SF-1 activates transcription from the Vanin-1 promoter via two binding sites, both of which are required for maximal activation and that the presence of SOX9 further stimulates promoter activation. These in vitro observations are supported by the in vivo expression analysis demonstrating that Vanin-1 is co-expressed with SF-1 in both the adrenal cortex and the Leydig and Sertoli cells of the testis. Levels of Vanin-1 protein within the adrenal gland appeared lower in comparison to the testis cords, probably because SOX9 is not expressed in the adrenal gland (12). The expression of Vanin-1 in Leydig cells was weaker at 13.5 dpc compared with 16.5 dpc, coincident with an increase in the expression level of SF-1 in the same period of testis development (6), further providing in vivo support for the in vitro dose-dependence reported in this study.

A mouse mutant lacking Vanin-1 function shows no defects in gonadal development or fertility (17). As Vanin-1 is a member of a family of pantetheinase enzymes (15), it is possible that other members of the Vanin family or other proteins with similar enzymatic activity can replace Vanin-1 activity in the homozygous null mice. However, this may not be the case in all tissues, because the family members are differentially expressed (15). The kidney and liver that were tested from these null mice were found to lack cysteamine, a product of Vanin-1 activity, suggesting that the various pantetheinases do not overlap functionally. In addition, other members of this family are secreted, whereas Vanin-1 is cell-associated (15, 17).

The potential roles that Vanin-1 is likely to play in testis and adrenal function are summarized in Fig. 7. Vanin-1 is one of a class of enzymes responsible for the catabolism of pantetheine, formed during degradation of coenzyme A (CoA), to produce pantothenate (vitamin B5) and cysteamine (36, 37). Pantothenate is converted to CoA by a crucial biosynthetic pathway. An essential co-factor in a large number of pathways including hormone synthesis, CoA is required for the initial steps of cholesterol synthesis, in particular for the activity of the essential enzyme 5-hydroxy-3-methylglutaryl-CoA reductase. The gene encoding this enzyme has also been identified as a regulatory target of SF-1 (38). Cholesterol is required for the synthesis of the steroid hormones produced by both the adrenal gland and the Leydig cells of the testis, and it has been shown experimentally that the presence of high levels of pantetheine can inhibit cholesterol synthesis (39). The presence of Vanin-1 and other pantetheinase enzymes to recycle pantetheine to pantothenate would reduce the levels of pantetheine and hence...
prevent inhibition of cholesterol synthesis, in addition to providing more pantothenate for CoA synthesis. This would suggest that in addition to up-regulating expression of steroid synthesis enzymes, SF-1 also targets genes required for optimal synthesis of metabolites in these pathways.

In addition to a likely role in steroid metabolism, increasing evidence supports a role for Vanin-1 in the cellular/tissue stress response. Alterations in the levels of reactive oxygen species present in tissues affect cellular proliferation, differentiation, and apoptosis. Cysteamine, a product of Vanin-1 activity, is a potential anti-oxidant and is lacking in the tissues that were analyzed from Vanin-1-deficient mice (19). Cysteamine has been shown to reduce radiation-induced sperm abnormalities in vitro (40) and to reduce the toxicity of various agents in Sertoli-germ cell co-cultures (41). The major role of Sertoli cells is to support the germ cells within the testis, and they are known to express high levels of enzymes that catalyze synthesis of antioxidants or scavenge reactive oxygen species such as glutathione S-transferase and superoxide dismutase (42–44). The presence of Vanin-1 on the surface of Sertoli cells is likely to produce high levels of cysteamine surrounding germ cells within the testis cords, which likely act as a protectant against reactive oxygen species. This degree of protection is likely much more important in the fetal testis compared with the fetal ovary, because the former undergoes much more rapid cellular proliferation and begins synthesis of steroid hormones therefore significantly increasing the generation of reactive oxygen species produced relative to the latter. Generation of antioxidants such as cysteamine will help to regulate the oxidative stress response during this high metabolic stage of gonad development and provide necessary protection from damage to the germ line.

In this context it is significant that detoxifying enzymes such as GSTs and superoxide dismutase are also present at high levels in the testis and have been shown to be an important part of protection of germ cells from oxidative stress-induced apoptosis (45). Vanin-1 knock-out mice have elevated glutathione stores because of reduced inhibition of γ-glutamylcysteine synthetase by cysteamine. Vanin-1 may modulate oxidative stress response through the glutathione pathway in the testis.

In addition, mice heterozygous for SF-1 mutations show impaired adrenal gland function under stress (46). This impaired stress response may be because of reduced expression of SF-1-regulated genes such Vanin-1 in these tissues, given that the Vanin-1 promoter has been shown to be up-regulated, through two antioxidant response elements, in response to oxidative stress. In vitro data suggested that these antioxidant response elements are occupied by AP-1 and SF-1 transcription factors, both of which have been implicated in the regulation of other SF-1 target genes. AP-1 sites are present in the StAR gene promoter (47, 48), whereas SF-1 is a general transcription factor regulation involved in the regulation of human StAR (49), rat luteinizing hormone β (50), and bovine cholesterol side chain cleavage enzyme (20) gene promoters along with SF-1. The mouse Vanin-1 gene promoter is thus another example of a promoter regulated by a combination of SF-1, AP-1, and SF-1 transcription factors.

We have shown that mouse Vanin-1 expression is regulated by SF-1 and SOX9. This is likely to contribute to regulation of the stress response in testis and maintaining CoA levels. Further investigation is underway to determine the exact functional role(s) of Vanin-1 and other pantetheinase enzymes in the development of the embryonic testis.

Acknowledgments—We thank Dagmar Wilhelm for technical expertise and, along with James Smith, for critical reading of the manuscript, Kristy James and Jo Bowles for purifying the Vanin-1 antibody, and Philippe Naquet for providing the H202-407-7-4 hybridoma cell line. We also thank Ken-Ichiro Morohashi for the kind gift of SF-1 antisera, Rohan Teasdale for bioinformatics assistance, and Richard Behringer for the Amhr2-Cre line.

REFERENCES
1. Bowles, J., Schepers, G., and Koopman, P. (2000) Dev. Biol. 227, 239–255
2. Vidal, V. P., Chabosse, L. M. C., de Rooij, D. G., and Schiegl, A. (2001) Nat. Genet. 28, 216–217
3. Foster, J. W., Dominguez-Steiglitz, M. A., Guidi, S., Kwok, C., Weller, P. A., Weissbach, J., Manour, S., Young, I. D., Goodfellow, P. N., Brook, J. D., and Schafer, A. J. (1994) Nature 372, 525–530

FIG. 7. Proposed role of Vanin-1 in Sertoli and steroidogenic cells based on its enzymatic activity. SF-1 has been found to up-regulate expression of enzymes required for both cholesterol and steroid hormone synthesis. Vanin-1 recycles pantetheine, produced by the degradation of CoA, to pantothenate to be reused in CoA synthesis. CoA is required for the early steps of cholesterol synthesis; cholesterol is a key component in the generation of steroid hormones. Production of cysteamine by Vanin-1 is likely to play an important role in regulating the oxidative stress response of these tissues in particular in the protection of the nearby germ cells from reactive oxygen species.
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4. Arango, N., Lovell-Badge, R., and Behringer, R. (1999) Cell 99, 409–419
5. de Santa Barbara, P., Moniot, B., Poulat, F., Boizet, B., and Berta, P. (1998) J. Biol. Chem. 273, 29654–29660
6. Hatano, O., Takayama, K., Imai, T., Waterman, M. R., Takakusu, A., Omura, T., and Morohashi, K. (1998) Development 125, 2787–2791
7. Ikeda, Y., Shen, W. H., Ingraham, H. A., and Parker, K. L. (1994) Mol. Endocrinol. 8, 654–662
8. Luo, X., Ikeda, Y., and Parker, K. L. (1994) Cell 77, 481–490
9. Reinhart, A. J., Williams, S. C., Clark, B. J., and Stocco, D. M. (1999) Mol. Endocrinol. 13, 729–741
10. de Santa Barbara, P., Moniot, B., Poulat, F., Boizet, B., and Berta, P. (1998) Mol. Endocrinol. 12, 1310–1320
11. Schepers, G., Wilson, M., Wilhelm, D., and Koopman, P. (2003) J. Biol. Chem. 278, 28101–28108
12. Shen, J. H.-C., and Ingraham, H. A. (2002) J. Biol. Chem. 277, 108–116
13. Bowles, J., Bullejos, M., and Koopman, P. (2000) Mol. Endocrinol. 14, 1553–1560
14. Grimmond, S., Van Hateren, N., Siggers, P., Arkell, R., Soares, M., Bonaldo, M., Smith, L., Tymowska-Lalanne, Z., Wells, C., and Greenfield, A. (2000) Hum. Mol. Genet. 9, 1553–1560
15. Martin, F., Malerque, F., Pitari, G., Philippe, J. M., Philips, S., Chabret, C., Granjeaud, S., Mattei, M. G., Mungall, A. J., Naquet, P., and Galland, F. (2001) Immunogenetics 53, 296–306M. F.
16. Maras, B., Barra, D., Dupre, S., and Pitari, G. (1999) FEBS Lett. 461, 149–152
17. Pitari, G., Malerque, F., Martin, F., Philippe, J., Massucci, M., Chabret, C., Maras, B., Dupre, S., Naquet, P., and Galland, F. (2000) FEBS Lett. 483, 149–154
18. Martin, F., Penet, M. F., Malerque, F., Lepidi, H., Desein, A., Galland, F., de Reggi, M., Naquet, P., and Gharbi, B. (2004) J. Clin. Investig. 113, 591–597
19. Berruyer, C., Martin, F. M., Castellano, R., Macone, A., Malerque, F., Gazzola-Urban, S., Millet, V., Imbert, J., Dupre, S., Pitari, G., Naquet, P., and Galland, F. (2004) Mol. Cell. Biol. 24, 7214–7224
20. Liu, Z., and Simons, E. R. (1999) Mol. Cell. Endocrinol. 153, 183–196
21. 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
22. Wilhelm, D., and Englert, C. (2002) Genes Dev. 16, 1839–1851
23. Mather, J. P. (1980) Biol. Reprod. 23, 243–252
24. Aurand-Lions, M., Galland, F., Babinet, H., Zakhayev, V., Imhof, B. A., and Naquet, P. (1996) Immunity 5, 391–405
25. Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996) Development 122, 2813–2822
26. Jeyasuria, P., Ikeda, Y., Jamin, S. P., Zhao, L., de Rooij, D. G., Themmen, A. P. N., Behringer, R. R., and Parker, K. L. (2004) Mol. Endocrinol. 18, 1610–1619
27. Morohashi, K., Honda, S., Inomata, Y., Handa, H., and Omura, T. (1992) Mol. Endocrinol. 14, 1235–1245