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Nuclear Receptor Steroidogenic Factor 1 Directs Embryonic Stem Cells toward the Steroidogenic Lineage

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The orphan nuclear receptor steroidogenic factor 1 (SF-1) is expressed in the adrenal gland and gonads and is an important regulator of the expression of cytochrome P-450 steroidogenic enzymes in cultured cells. Targeted disruption of the SF-1 gene in mice shows that it is a critical participant in the genetic program that promotes the development of urogenital mesoderm into the adrenal gland and gonads. To assess the ability of SF-1 to regulate this differentiation pathway, we ectopically expressed SF-1 in murine embryonic stem (ES) cells. We found that stable expression of SF-1 is sufficient to alter ES cell morphology, permit cyclic AMP (cAMP) and retinoic acid-induced expression of the endogenous side chain cleavage enzyme gene, and consequently, promote steroidogenesis. While steroid production is dependent upon SF-1, cAMP induction of steroidogenesis does not enhance the responsiveness of an SF-1-specific reporter. Furthermore, the activity of a $P450_{SCC}$ promoter/luciferase reporter construct, which is induced by cAMP in steroidogenic cells and ES cells converted by stable expression of SF-1, is not induced by cAMP in wild-type ES cells transiently transfected with SF-1, suggesting that the induction of downstream gene products is required before steroidogenesis can occur. We demonstrate that mutants which disrupt the DNA binding domain or the AF2 transcriptional activation domain of SF-1 do not confer the steroidogenic phenotype to ES cells. Interestingly, however, AF2 mutants fused to the VP16 activation domain do confer the steroidogenic phenotype to ES cells, but only in the presence of a portion of the ligand binding domain. These studies extend the role of SF-1 in steroidogenic tissues to that of a dominant regulator of the steroidogenic cell phenotype.

The steroid receptor superfamily of transcription factors is a diverse set of proteins with clear influence over developmental, physiological, and neoplastic processes. The majority of these proteins share structural motifs that guide their function: an amino-terminal activation domain (domains A/B), a central DNA binding domain (DBD) (domain C), an intervening hinge region (domain D), and a carboxy-terminal ligand binding domain (LBD) (domain E), which mediates ligand-induced transactivation and participates in receptor dimerization (52). Steroidogenic factor 1 (SF-1) is an orphan member of this family, as no ligand has yet been found to modulate its activity. It is further classified as a monomeric nuclear receptor, as it is able to bind to a single estrogen receptor half-site element (5′-AGGTCA) preceded by the preferred nucleotides 5′-TCA (52, 74). SF-1 is constitutively expressed in all three layers of the adrenal cortex, in Leydig and Sertoli cells of the testis, in ovarian granulosa and theca cells, in the placenta, as well as in the pituitary and hypothalamus (35, 68). SF-1 has been shown to regulate the expression of each of the steroidogenic cytochrome P-450 enzyme genes (2, 16, 30, 33, 45, 51, 53, 57, 81), Mullerian inhibitory substance (69), and the α- and β-subunits of the gonadotropins (5, 27, 38, 42, 46), suggesting that SF-1 has a central role in the regulation of steroidogenesis, development, and reproduction.

SF-1 is the mammalian homolog of FTZ-F1, an orphan nuclear receptor that is a critical regulator of embryogenesis in *Drosophila* (26, 79). The significance of SF-1 in mammalian development is supported by its expression in the mouse urogenital ridge as early as embryonic day 9 (E9), before distinct adrenocortical and gonadal anlagen can be distinguished (35). Moreover, disruption of the SF-1 gene in mice demonstrates that it is required for the formation of the adrenal glands and gonads; SF-1 (−/−) mice completely lack these structures, and as a result of gonadal agenesis, genotypically male SF-1 (−/−) mice retain Mullerian structures (50, 68). Microscopic analysis of targeted embryos demonstrates that the primitive adrenals and gonads initially form but then undergo degeneration by E13 via cellular apoptosis. All SF-1 (−/−) mice die within a few hours of birth unless supported with exogenous steroids (50). Detailed analysis of the hypothalamus demonstrates that the ventromedial hypothalamic nucleus also fails to appropriately develop in SF-1 (−/−) mice (34). These studies clearly demonstrate that SF-1 is a critical component in the genetic events which guide the formation of endocrine tissues.

While the analysis of SF-1 (−/−) mice demonstrates the integral role of SF-1 in the ontogeny of steroidogenic tissues, the complete absence of steroidogenic organs in SF-1 (−/−) mice raises new questions with regard to the development and function of these organs. For example, the mechanism(s) through which SF-1 permits the steroidogenic tissues to differentiate and become functional is not known. The ultimate regulation of steroidogenesis presumably requires a host of transcription factors including SF-1 and downstream target genes to mediate appropriate responsiveness of steroidogenic enzymes as well as cholesterol mobilization pathways to hormones such as adrenocorticotropin (ACTH) and gonadotropins (62, 64). These hormones enhance steroidogenesis through cyclic AMP (cAMP) as a second messenger, but retinoids such as all-*trans*-retinoic acid (all-*trans*-RA) are also known to enhance steroidogenesis (14, 41, 64, 70). While the action of cAMP has been shown in some cases to proceed through SF-1 and the cAMP response element-binding protein CREB (9), several steroidogenic enzyme gene promoters lack

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canonical cAMP response elements. Moreover, cAMP induction of several steroidogenic gene promoters occurs independently of SF-1 binding sites (10, 13). Thus, the precise mechanisms that direct both tissue-specific and hormone-inducible steroidogenic enzyme gene expression remain elusive.

As SF-1 (−/−) mice lack primary steroidogenic tissues that would otherwise permit sophisticated analysis of these mechanisms, another model system is required. In this report we demonstrate that stable expression of SF-1 in murine embryonic stem (ES) cells initiates a genetic program that shifts cell fate toward a steroidogenic phenotype. Despite the continual presence of feeder cells and leukemia inhibitory factor (which prevent differentiation), ES cells stably expressing SF-1 have altered morphology and respond to cAMP and RA by induction of the side chain cleavage ($P450_{SCC}$) gene, which encodes the rate-limiting steroidogenic enzyme. Consequently, these agents induce progesterone production in cells stably expressing SF-1 but not wild-type ES cells. While SF-1 is indispensable for this conversion to take place, SF-1 itself is not directly influenced by cAMP and RA. These data suggest that stable expression of SF-1 initiates a genetic program that drives ES cell fate towards the steroidogenic lineage, and the subsequent induction of additional gene products confers responsiveness to cAMP and RA. Thus, the role of SF-1 in vivo is refined from a critical component in development to a true effector of cell fate within steroidogenic tissues.

MATERIALS AND METHODS

ES cell culture, stable transfection, and hormone assays. RW4 mouse strain 129/SvJ ES cells were maintained on a feeder layer of murine embryonic fibroblasts (MEFs) (γ-irradiated with 3,000 rads to induce growth arrest) in Dulbecco's modified Eagle's medium with 15% fetal bovine serum, 100 μM 2-mercaptoethanol, 1,000 U of leukemia inhibitory factor per ml, and antibiotics. Transgenic lines which express PGK-neo-bpA or PGK-puro-pA were used to generate MEFs that are resistant to G418 and puromycin, respectively. For stable transfection, 5×10^6 ES cells were electroporated with 25 μg of plasmid DNA (liberated from vector sequences by restriction digestion) in 1× phosphate-buffered saline with a BTX 300 transfector (Biotechnologies and Experimental Research Inc., San Diego, Calif.) by applying 195 V at 500 μF. Transfected cells were plated to a confluent layer of MEFs, and 300 μg of G418 (Calbiochem [San Diego, Calif.] catalog no. 345810-R) or 1 μg of puromycin (Sigma Chemical Co. [St. Louis, Mo.] catalog no. P7255) per ml was administered starting the following day for 5 days. On the 6th day, colonies were picked and expanded, while being maintained on MEFs in standard ES media. ES cells were examined and photographed by phase-contrast luminescence using a Nikon TMS-F inverted-microscope/camera assembly.

For stimulation experiments, ES cells were plated at 5×10^4 per well in 24-well plates in 0.5 ml of ES medium. After 1 day, cells were given 5 μg of 20α-hydroxycholesterol (Sigma, H6378) per ml in the presence or absence of 1 mM 8Br-cAMP (Sigma, B7880) and/or 0.5 μM all-*trans* RA (Sigma, R2625). After 48 h, media were harvested and assayed for progesterone by radioimmunoassay using the Coat-a-Count system (Diagnostic Products Corporation, Los Angeles, Calif.). Inter- and intraassay coefficients of variation were 5.1 and 2.6%, respectively.

Plasmid constructions. All constructs which required the PCR utilized the high-fidelity KlenTaq polymerase enzyme (4). All expression vectors used for ES cell transfections are derivatives of the vector pCAGGS (37) (a gift from M. Sands, Washington University), which uses the cytomegalovirus (CMV) immediate-early enhancer and the chicken β-actin promoter and first intron enhancer. Expression vectors used for JEG-3 cell transfections were constructed with pCMVneo (7). The PGK-neo-bpA cassette (a gift from A. Bradley) was cloned as an *Xho*I fragment into the *Sal*I site of pCAGGS to generate β-actin-neo. The PGK-puro-pA cassette was generated by replacing the simian virus 40 early promoter with the PGK promoter in pPUR (Clontech, Palo Alto, Calif.). This 1.6-kb cassette was amplified by PCR using *Xho*I-tagged primers flanking the promoter and pA signal, cut with *Xho*I, and cloned into the *Sal*I site of pCAGGS to generate β-actin-puro. The SF-1 cDNA was ligated into each of these vectors by using *Eco*RI on both vector and insert. SF-1 (mDBD) was generated by inverse PCR mutagenesis of the SF-1 cDNA in pBSKS (Stratagene, La Jolla, Calif.) using forward primer 5′-CTGCTCACGTGGGAGATCTTCAAGGGC-3′ and reverse primer 5′-CCCGTAGTGGTAGCCCGACACC-3′ (underlined letters indicate nucleotides changed to mutate P-box cysteines). The SF-1 (mDBD) cDNA was then ligated into β-actin-neo. SF-1 (mAF2) was generated by PCR mutagenesis using a primer that mutagenized codons 456 (Met to Ala) and 457 (Leu to Ala). This PCR product was ligated into β-actin-neo. The NGFI-B cDNA was digested from CMV-NGFI-B and ligated into β-actin-neo. The LRH cDNA (a gift from S. A. Kliewer, Glaxo Research Institute) was cut out of pBSKS ligated into β-actin-neo.

The full-length SF-1 cDNA was cloned into pCMVneo to generate CMV-SF-1 (wt) as previously described (75). CMV-SF-1 (mAF2) was generated by ligating SF-1 (mAF2) cDNA into pCMVneo. The SF-1 (ΔAF2) cDNA truncation product was generated by PCR amplification using a primer that introduces a stop codon 5′ to AF2. The resulting product was digested and ligated into pCMVneo. The SF-1 fragments encoding residues 1 to 104 (DBD) and 1 to 277 were amplified by PCR using the same forward primer which spans the initiation codon. The reverse primer used for SF-1 (1-277) was a primer that introduces a stop codon after codon 277, and that used for SF-1 (DBD) was a primer that introduces a stop codon after codon 104. The resulting fragments were digested and ligated into pCMVneo, which generated CMV-SF-1 (1 to 277) and CMV-SF-1 (DBD), respectively. Residues 411 to 487 of the VP16 activation domain were amplified from the plasmid pUHC15-1 (25) (tetR-VP16 fusion) containing the VP16 activation domain from herpes simplex virus 1.1 by using a forward primer that includes a Kozak initiation signal and a reverse primer. This ~240-bp product was digested and ligated upstream of SF-1 into CMV-SF-1 (wt), CMV-SF-1 (ΔAF2), CMV-SF-1 (1-277), CMV-SF-1 (DBD), or CMV-VP16-SF-1 (DBD) to generate the appropriate in-frame fusions of VP16 and SF-1 in pCMVneo. For ES cell transfections, each of the entire VP16-SF-1 fusion cassettes was digested from pCMVneo and ligated into β-actin-puro. Each construction was sequenced on automated sequencer ABI 373A (Applied Biosystems, Inc., Foster City, Calif.) For each of the β-actin-neo expression constructs, vector sequences were removed by digestion with *Hin*dIII before transfection, and for each of the β-actin-puro constructs, vector sequences were removed with *Bam*HI.

Northern analysis. Northern blots were performed essentially as previously described (18). For SF-1, the probe was generated by PCR and consisted of the first 300 nucleotides of the mouse coding sequence. For $P450_{SCC}$, the probe was generated by RT-PCR of mouse adrenal gland RNA and consisted of the first 345 nucleotides of the coding sequence. For cyclophilin, a 700-bp probe was used from the rat cDNA. After a wash, membranes were exposed to a PhosphorImager screen, scanned, and quantitated by the ImageQuant software package (Molecular Dynamics, Sunnyvale, Calif.). Where appropriate, quantitation was performed relative to ethidium bromide fluorescence of 28S rRNA bands (quantitated by densitometry using the IS-1000 software package [Alpha-Innotech Corporation, San Leandro, Calif.]) and was confirmed against cyclophilin expression.

Transient transfections. JEG-3 human choriocarcinoma cells were cultured in minimal essential medium with Earle's salts, 10% fetal bovine serum, and antibiotics, as described elsewhere (17). For transfection, cells were seeded at 10^5 per well of a six-well plate and transiently transfected the following day with a total of 2 μg of plasmid DNA including 0.5 μg of reporter (either SF-1/Luc, which consists of two SF-1 consensus binding sites [5′-TCAAGGTCA] spaced with 5 nucleotides, cloned 5′ to the minimal prolactin promoter TATA box driving luciferase, or hSCC/Luc, which consists of 2.3 kb of the human $P450_{SCC}$ proximal 5′-flanking region driving luciferase, a gift from W. Miller, University of California, San Francisco [56]), 100 ng of expressor, and 200 ng of CMV-β-gal to correct for transfection efficiency and cell number. Forty-eight hours after transfection, cells were lysed in a buffer containing 1% Triton X-100 and assayed for luciferase activity and β-galactosidase activity as previously described (75). All samples were transfected in duplicate at least three times.

For ES cell transient transfections, 10^5 cells per gelatinized well of a six-well plate were seeded, and 2 days later 3 μg of plasmid DNA was transfected by the Lipofectin reagent (Gibco BRL, Grand Island, N.Y.) according to the manufacturer's instructions. Two micrograms of SF-1/Luc or hSCC/Luc reporter was added along with 500 ng of expressor and 500 ng of RSV-βgal for control as above. After an overnight incubation with the liposome-DNA mixture, cells were fed with ES media with or without 1 mM 8Br-cAMP. Forty-eight hours after addition of DNA, cells were harvested and analyzed as above. For a standardized number of cells, activities from the RSV-βgal reporter in native ES cells and SF-1-converted ES cells were equivalent.

Electrophoretic mobility shift assay. This assay was performed essentially as described elsewhere (74). Briefly, proteins were prepared by using the TnT in vitro transcription/translation kit (Promega, Madison, Wis.), plasmid pBSKS, and T3 or T7 RNA polymerase, as appropriate. Proteins were incubated with double-stranded, [γ-^{32}P]ATP-labeled oligonucleotide fragments containing the 5′-TCAAGGTCA-3′ SF-1 binding site as previously described (74) and run on a 6% polyacrylamide gel. After drying, the gel was exposed to a PhosphorImager screen, and data were analyzed as above.

RESULTS

Wild-type SF-1, but not DBD or AF2 mutants, alters ES cell morphology. Undifferentiated ES cells are derived from the bilayered inner cell mass of blastocyst stage embryos (66). When grown on MEF feeder cells at a low passage number, they organize as spherical colonies which are transilluminated

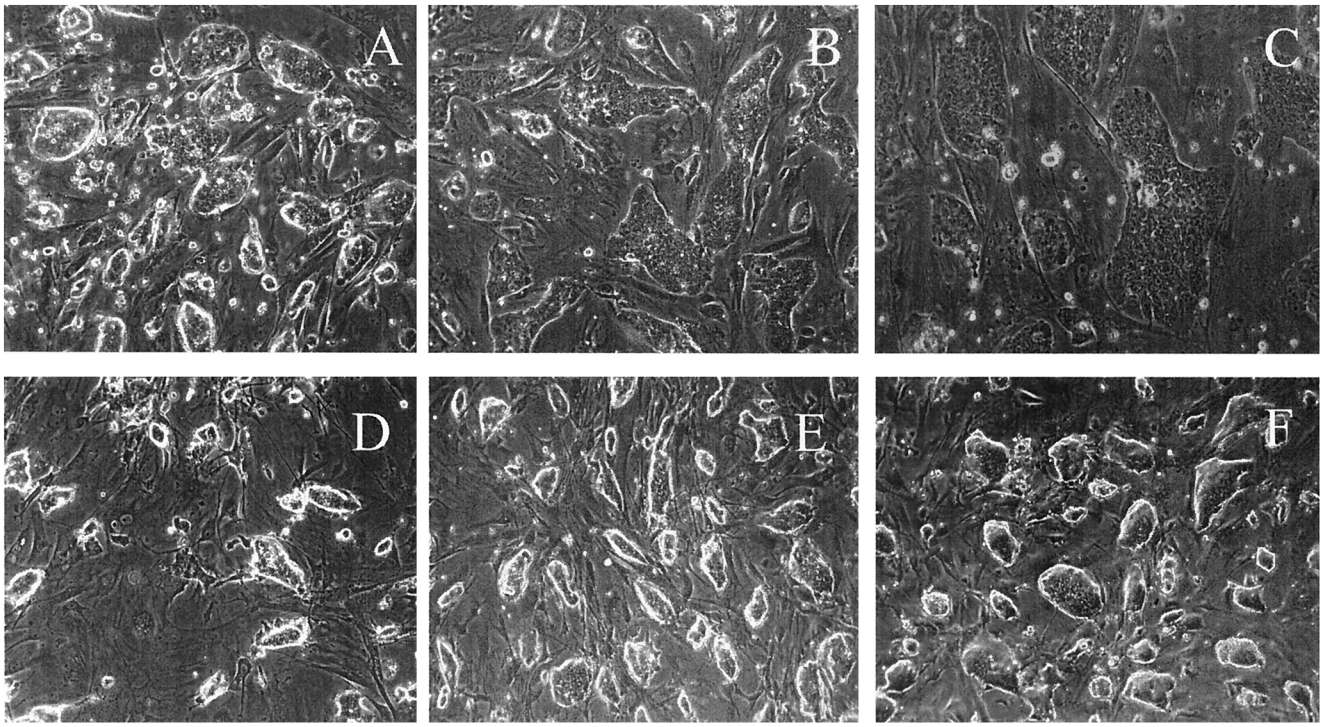

FIG. 1. Stable expression of SF-1 alters ES cell morphology. Native undifferentiated ES cells were stably transfected with expression vectors. After selection, isolated clones were expanded under nondifferentiating conditions (see Materials and Methods). Photomicrographs were taken under phase-polarized light. (A) Native ES cells; (B and C) ES cells stably transfected with SF-1 (wt); (D) empty expression vector; (E) SF-1 (mDBD); (F) SF-1 (mAF2). Original magnification, ×100 (A, B, and D to F) or ×200 (C).

under phase-polarized light, consequently obscuring the morphology of individual cells (Fig. 1A). Undifferentiated wild-type RW4 ES cells were stably transfected with SF-1 (wt) driven by the chicken β-actin promoter and selected with G418 on MEFs which harbor a PGK-neo resistance cassette. Immediately upon selection of resistant colonies, we noticed an altered morphology of colonies that were transfected with SF-1 (Fig. 1B and C) but not of cells transfected with the empty vector (Fig. 1D). Instead of growing as birefringent spheres, all SF-1-transfected colonies grew as flat, phase-dull sheets in which individual cells are easily discriminated.

To determine if this morphological conversion required domains known to be critical for SF-1 function, we stably transfected two SF-1 mutants. SF-1 (mDBD) contains mutated cysteines within the P box of the DBD, rendering it unable to form zinc fingers and therefore incapable of binding DNA. This mutant does not alter ES cell morphology (Fig. 1E), in contrast to that observed with SF-1 (wt). SF-1 (mAF2) changes the last two hydrophobic residues of SF-1's AF-2 domain. This domain forms an α-helical structure that is required for ligand-induced transactivation by many nuclear receptors (6, 19, 47). It is also required for appropriate transcriptional activation by SF-1, as its mutation or elimination equally reduces transcriptional activity (17; also, see below). SF-1 (mAF2) is also incapable of converting the morphology of ES cells (Fig. 1F). While wild-type ES cells do not normally express SF-1, the transfected wild-type and mutant SF-1 transgenes are all expressed (Fig. 2). To further demonstrate the specificity of this phenomenon, we transfected ES cells with the cDNA encoding LRH, which is a nuclear receptor that is 90% identical to SF-1 within the DBD and 100% identical within AF2, and found that this gene does alter ES morphology in a manner similar to SF-1 (data not shown). Finally, we transfected the cDNA for NGFI-B, which is a related orphan nuclear receptor that is a potent activator of transcription as a monomer but binds a slightly different response element than SF-1 by virtue of its distinct A-box region within the DBD (74). Transfection of NGFI-B does not alter ES morphology (data not shown). Therefore, the

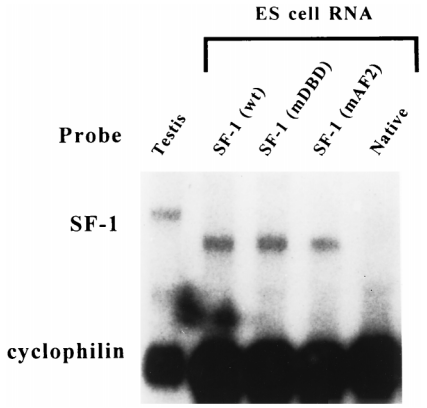

FIG. 2. Wild-type or mutated SF-1 is expressed in stably transfected but not native ES cells. A Northern blot demonstrates the expression of SF-1 constructs (see Materials and Methods) in native ES cells. A ^{32}P-labeled SF-1 probe was generated and hybridized as described in Materials and Methods. Note the absence of SF-1 expression in native ES cells and the altered mobility of the transgene transcript compared to that of the endogenous SF-1 transcript present in testis RNA, due to the use of heterologous 5′- and 3′-untranslated vector-derived sequences in the transgene construct that are shorter than those of the endogenous SF-1 transcript. RNAs were shown to be equally loaded by probing for cyclophilin.

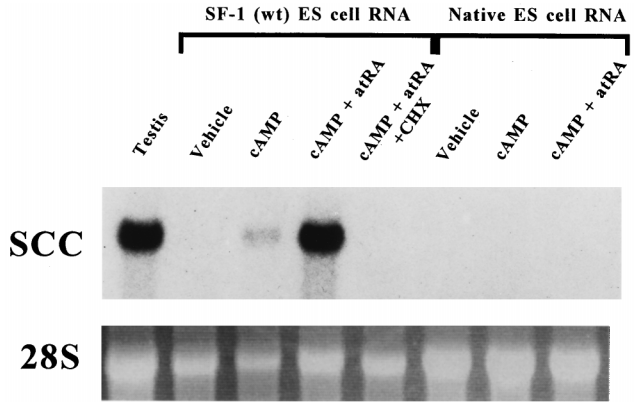

FIG. 3. SF-1-expressing ES cells express $P450_{SCC}$ (side chain cleavage) mRNA. A Northern blot demonstrates expression of $P450_{SCC}$ (SCC) in ES cells stably expressing wild-type SF-1 but not in native ES cells. Where indicated, cells were treated with 1 mM 8Br-cAMP, 0.5 μM all-*trans*-RA (atRA), and/or 10 μg of cycloheximide (CHX) per ml for 20 h before harvest. The 28S rRNA band is presented to illustrate equivalent loading of the RNAs and was used for standardization.

conversion of ES morphology from an undifferentiated, pluripotent cell to that of a more differentiated ES cell by SF-1 specifically requires the DNA binding and transcriptional activation capacities of the molecule and thus reflects a specific nuclear receptor-mediated phenomenon.

ES cells differentiated by SF-1 express the endogenous side chain cleavage gene and generate progesterone. To determine if the converted ES cell morphology corresponds to a definable differentiation event, we examined the expression of a target gene for SF-1, $P450_{SCC}$. For these experiments we utilized 8Br-cAMP (cAMP) and all-*trans*-RA, which are agents that induce steroidogenesis in several steroidogenic cell lines (see the introduction). Native ES cells and three isolated clones of SF-1-converted ES cells were grown to confluence and then treated with vehicle or a combination of these agents for 20 h. A representative Northern blot on isolated RNAs demonstrated that the $P450_{SCC}$ transcript is expressed in ES cells stably expressing SF-1 but not native ES cells. $P450_{SCC}$ expression in SF-1-converted cells is clear after induction with cAMP (7-fold over unstimulated SF-1-converted ES cells) and is greatly increased (35-fold over unstimulated) by treatment with both cAMP and RA. Native ES cells and ES cells stably transfected with SF-1 (mDBD) or SF-1 (mAF2) do not express the $P450_{SCC}$ gene, even after treatment with cAMP and RA (Fig. 3 and data not shown). Importantly, the induction of $P450_{SCC}$ in SF-1-converted cells is dependent upon ongoing protein synthesis, as addition of 10 μg of cycloheximide per ml to cells treated with cAMP or both cAMP and RA completely abrogates enhanced expression of $P450_{SCC}$ mRNA (standardized signal is equivalent to unstimulated SF-1-converted ES cells) (Fig. 3). Thus, the induction of $P450_{SCC}$ by cAMP and/or RA in ES cells stably expressing SF-1 may require the induction of additional downstream regulators.

To ascertain if this induced expression of the rate-limiting enzyme $P450_{SCC}$ coordinately increases progesterone synthesis, we assayed media conditioned by wild-type and SF-1-converted ES cells in the presence or absence of cAMP and/or RA. All cells were cotreated with 5-μg/ml 20α-hydroxycholesterol, which bypasses the outer mitochondrial membrane and thus serves as a more potent substrate than cholesterol for $P450_{SCC}$ action on the inner membrane (49). cAMP and RA induced all three clones of SF-1-converted, but not wild-type, ES cells to produce progesterone, as assayed by a progesterone-specific radioimmunoassay. When given together, cAMP and RA induced progesterone levels up to ~35 ng/ml (~50-fold induction) (Fig. 4). Native ES cells and cells stably transfected with SF-1 (mDBD) or SF-1 (mAF2) yielded progesterone levels below the assay's limit of sensitivity (0.1 ng/ml) and were not inducible with cAMP and RA (data not shown). Furthermore, cells stably expressing LRH generate progesterone to degrees similar to cells expressing SF-1, but ES cells expressing NGFI-B do not generate progesterone (data not shown). Thus, the morphological alteration, inducible expression of $P450_{SCC}$, as well as the inducible production of progesterone are all specifically dependent on wild-type SF-1 family nuclear receptor function.

cAMP-enhanced expression of side chain cleavage does not result from augmented SF-1 activity. To determine if the cAMP- and RA-enhanced expression of $P450_{SCC}$ and steroidogenesis in SF-1-converted ES cells is mediated directly through enhanced SF-1 action, we transiently transfected a synthetic SF-1 luciferase reporter (SF-1/Luc) into native wild-type and SF-1-converted ES cells and measured its inducibility by cAMP. Because this reporter gene contains only two SF-1 binding sites and a TATA box, it specifically monitors SF-1 activity. While activity of SF-1/Luc was ~18-fold higher in SF-1-converted ES cells than in native ES cells, its activity was not induced by cAMP in either cell line (Fig. 5A). SF-1/Luc activity in native cells transiently cotransfected with SF-1 also is not induced by cAMP, although SF-1 does increase SF-1/Luc activity ~23-fold. Therefore, SF-1 activity does not appear to be enhanced directly by cAMP, despite enhanced $P450_{SCC}$ expression and progesterone synthesis in response to cAMP.

We next sought to determine the ability of SF-1 to influence cAMP inducibility in a natural promoter context where the effects of additional transcription factors would be manifest. We transiently transfected SF-1-converted ES cells with hSCC/Luc, which harbors 2.3 kb of the human $P450_{SCC}$ promoter driving a luciferase reporter gene (56), and found that its activity is 3.4-fold inducible by cAMP (Fig. 5B). This reporter

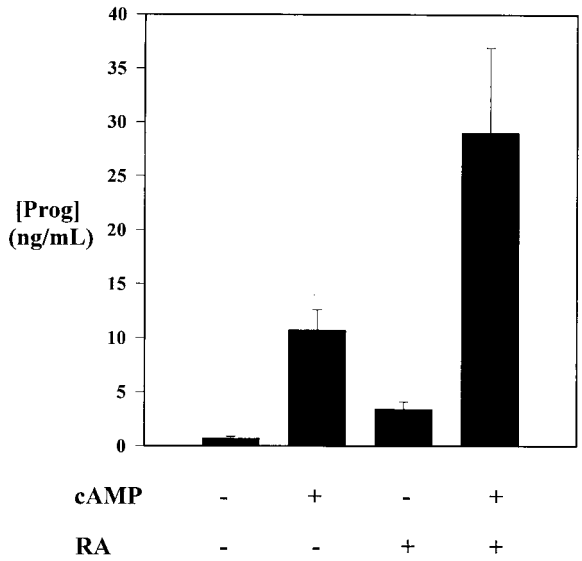

FIG. 4. Progesterone is produced by ES cells stably expressing SF-1. SF-1 (wt)-expressing ES lines were treated as described in Materials and Methods. Progesterone levels [Prog] were measured in the conditioned media. The data are means with standard deviations for seven independent experiments with three independent clones.

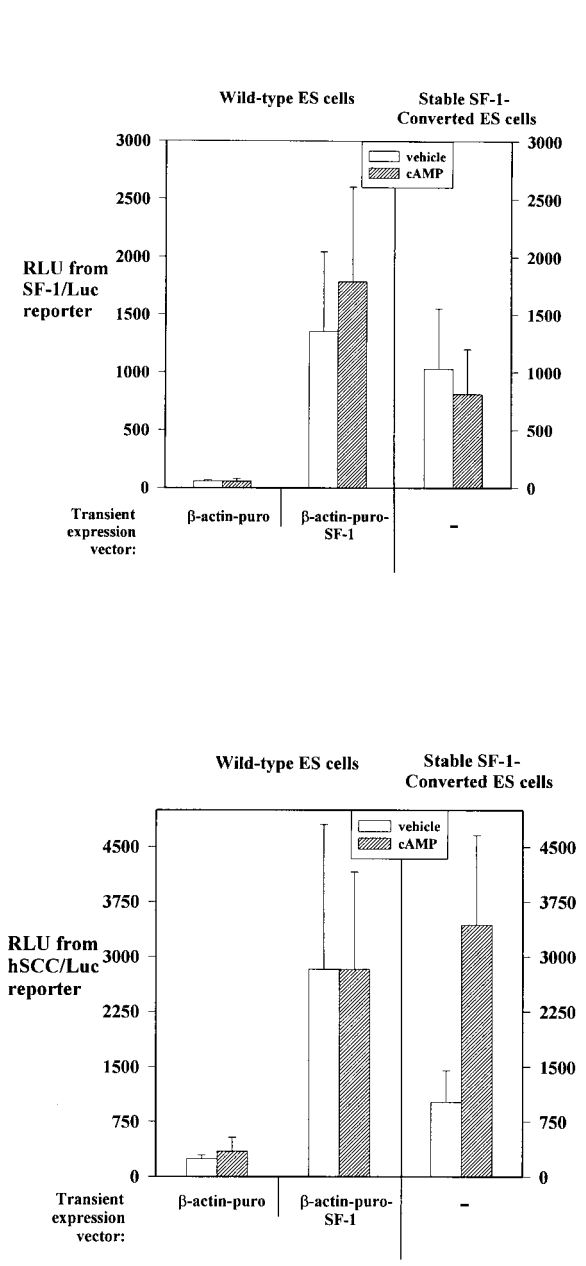

FIG. 5. cAMP induction of steroidogenesis in SF-1-expressing ES cells does not enhance SF-1 transcriptional activity. Transient transfection of ES cells was performed as described in Materials and Methods. The luciferase activity obtained from reporter plasmids was measured from cells that were treated with water vehicle or 8Br-cAMP. Native wild-type ES cells were cotransfected with a reporter construct and either an empty expression vector or an SF-1 expression vector, while ES cells converted by stable SF-1 expression were transfected with reporter only. The data are mean relative luciferase units (RLU) with standard deviations for at least four independent experiments, standardized against the β-galactosidase activity from a cotransfected RSV-βgal plasmid as described in Materials and Methods. (A) Activity obtained with SF-1/Luc, which contains two SF-1 binding sites and a TATA box in its promoter; (B) corresponding activities from hSCC/Luc, in which the luciferase reporter is driven by 2.3 kb of proximal 5′-flanking DNA from the human $P450_{SCC}$ gene (see Materials and Methods).

is not induced by cAMP in native ES cells. As with the SF-1/Luc reporter, transient cotransfection of native cells with SF-1 increases promoter activity of hSCC/Luc (~12-fold), but cAMP does not further enhance activity. Thus, the induction of $P450_{SCC}$ by cAMP in SF-1-converted ES cells does not occur through SF-1 action alone, as its transient presence is not sufficient for cAMP inducibility. Taken together, these results suggest that stable expression of SF-1 alters the ES cell milieu in such a way as to allow cAMP inducibility of the endogenous or transfected $P450_{SCC}$ gene.

Conversion of ES cells by SF-1 does not specifically require AF2 but does require the proximal ligand binding domain. Preliminary mutagenesis experiments demonstrated that conversion of ES cells to steroidogenic cells by SF-1 required an intact DBD and AF2 domain (Fig. 1). To further determine the regions of SF-1 that are required for ES cell differentiation and to assess whether AF2 mutations could be rescued by a heterologous activation domain, we generated truncations of the SF-1 molecule and fused them to the VP16 activation domain. We first assessed the ability of these mutants to activate reporters after transient transfection into JEG-3 human choriocarcinoma cells. Cotransfection of these constructs with the SF-1/Luc reporter demonstrates that various truncations of the SF-1 LBD up to the DBD's A box (residue 104) results in a gradual loss of transactivation function that can be rescued, and even dramatically enhanced, by fusion to VP16. Interestingly, fusion of another copy of the VP16 activation domain to the weak activator VP16-SF-1 (DBD), to generate $VP16^2$-SF-1 (DBD), can restore activity levels to those above wild-type SF-1 on the SF-1/Luc reporter (~947- versus ~136-fold) (Fig. 6C). The relative activity of each of these constructs is replicated when the hSCC/Luc reporter is used (Fig. 6D). One notable exception, however, is the $VP16^2$-SF-1 (DBD) construct. In contrast to its high activity on SF-1/Luc, its activity is considerably lower than that of wild-type SF-1 on the hSCC/Luc reporter (~45- versus ~352-fold) (Fig. 6D). Importantly, the SF-1 (DBD) construct was shown to be competent to bind a consensus SF-1 binding site (5′-TCAAGGTCA) by electrophoretic mobility shift assay (Fig. 6B). Any loss of transcriptional activity is therefore not a result of diminished DNA binding. These results indicate that the AF2 domain of SF-1 is not specifically required to transcriptionally activate the $P450_{SCC}$ promoter, as the ΔAF2 mutant can be rescued by the addition of the VP16 activation domain. However, a portion of the ligand binding domain does appear to have an obligatory role in this process, as the DBD in the context of a very potent concatamerized VP16 activation domain only weakly transactivates the $P450_{SCC}$ promoter.

To assess the differentiative ability of each of the SF-1 mutants, we stably transfected them into ES cells. The results of stable transfections of wild-type SF-1 and all mutants examined in this study are presented in Table 1. In each case, the ability to morphologically convert the cells segregated with capacity to generate progesterone in a cAMP-inducible manner. While SF-1 (wt), VP16-SF-1, VP16-SF-1 (ΔAF2), and VP16-SF-1 (1-277) were able to differentiate ES cells, SF-1 (mDBD), SF-1 (mAF2), SF-1 (1-277), VP16-SF-1 (DBD), and, importantly, $VP16^2$-SF-1 (DBD) could not. To confirm appropriate expression of the latter construct, we performed Northern analysis in stably selected ES cells (Fig. 7A). We also performed transient cotransfections of native ES cells with a $VP16^2$-SF-1 (DBD) expression vector and a SF-1/Luc or hSCC/Luc reporter (Fig. 7B). As expected, $VP16^2$-SF-1 (DBD) activated SF-1/Luc ~12-fold, but hSCC/Luc was not induced, as it was when wild-type SF-1 was cotransfected (Fig. 5B). The inability of $VP16^2$-SF-1 (DBD) to convert ES cells is intriguing, because it is a potent activator of the SF-1 reporter SF-1/Luc (Fig. 6A). However, the inability of this construct to differentiate ES cells is more closely paralleled by its inability to induce the hSCC/Luc reporter in ES and JEG-3 cells. These results indicate that a critical region is harbored between residue 105

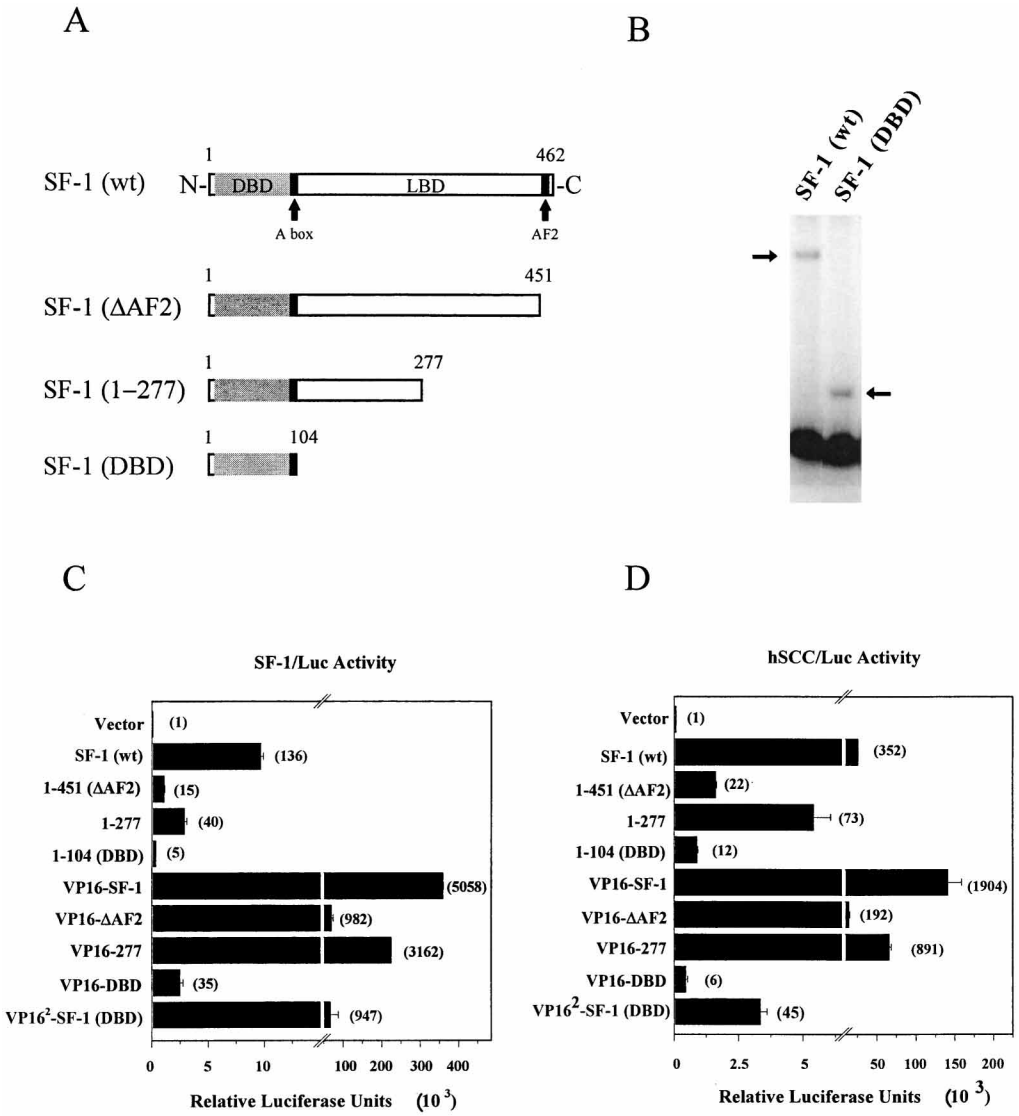

FIG. 6. Multiple domains are required for potent transcriptional activity on both a synthetic SF-1-responsive reporter and a $P450_{SCC}$ promoter reporter. (A) Schematic diagram of SF-1 (wt) and mutant constructs. LBD, ligand binding domain. Numbers correspond to the SF-1 N-terminal and C-terminal residues for each construct. Where appropriate, the VP16 activation domain was fused upstream of SF-1 coding sequences. (B) Electrophoretic mobility shift assay of in vitro-translated wild-type and mutant constructs demonstrating DNA binding competence by SF-1 (DBD) compared to SF-1 (wt) binding. Note that the migration of each complex is different due to the dramatically different sizes of the input proteins (arrows). (C and D) Transient cotransfection of JEG-3 human choriocarcinoma cells with SF-1 expression vectors and reporters (see Materials and Methods). The data are means with standard deviations for duplicates from one representative experiment (of five experiments), standardized to the β-galactosidase activity obtained from a cotransfected CMV-βgal plasmid. (C) Transcriptional activity of the indicated SF-1 mutants obtained with the SF-1-specific reporter SF-1/Luc; (D) Corresponding activities from a $P450_{SCC}$-promoter/reporter, hSCC/Luc. Note similar relative fold activities of the mutants on both reporters (indicated in parentheses), except for the $VP16^2$-SF-1 (DBD) mutant.

and residue 277 within the LBD that specifies a function distinct from transcriptional activation capacity and DNA binding. Therefore, the ability of SF-1 to convert ES cells into steroidogenic cells requires an intact DBD, a potent activation domain, and a portion of the ligand binding domain, which may mediate an interaction with other regulatory factors.

DISCUSSION

SF-1 is an orphan member of the nuclear receptor superfamily of transcription factors that transactivates each of the cytochrome P-450 steroidogenic enzyme promoters in cultured cells (2, 16, 30, 33, 45, 51, 53, 57, 81). Analysis of mice targeted for disruption of the SF-1 gene indicates that SF-1 is an integral participant in the developmental cascade that guides steroidogenic tissue differentiation (50, 68). Loss-of-function studies illustrate that several other transcription factors, such as WT-1, Hox11, GATA-1, Pit-1, insulin promoter factor 1, c-Jun, AML1, and PU.1 are also required in mice for the development of an organ or specific cellular compartment (23, 29, 40, 44, 48, 60, 61, 65). The results presented here demonstrate not only that SF-1 participates in development but that it is autonomously capable of initiating a program that converts an ES cell to a steroidogenic cell. The ability of transcription factors to serve as master regulators has been observed in other organ systems as well, as all four of the myogenic basic helix-loop-helix transcription factors—MyoD, myogenin, MRF4, and Myf-5— have been shown to stimulate skeletal

TABLE 1. SF-1 constructs and their capacity to differentiate ES cells upon stable selection

Construct	Morphological change	Progesterone (ng/ml)[a]		Fold stimulation
		Unstimulated	+ cAMP	
SF-1 (wt)	+	0.70	10.69	15.3
SF-1 (mDBD)	−	—	—	
SF-1 (mAF2)	−	—	—	
SF-1 (1-277)	−	—	—	
VP16-SF-1	+	0.21	15.40	73.3
VP16-SF-1 (ΔAF2)	+	0.23	0.94	4.1
VP16-SF-1 (1-277)	+	0.31	5.52	17.8
VP16-SF-1 (DBD)	−	—	—	
$VP16^2$-SF-1 (DBD)	−	—	—	
Vector	−	—	—	

[a] Determined as described in Materials and Methods. —, below the assay's detection limit (0.1 ng/ml). The data are means for at least three trials with at least two clones of each construct.

muscle formation in the embryonic fibroblast line 10T1/2 (reviewed in reference 20). Detailed analysis of double-mutant mice has demonstrated that Myf-5 and MyoD normally act upstream of myogenin in a cascade which ultimately generates skeletal muscle (28, 59, 67, 73). Adipogenesis also follows such a cascade, as transfection studies with NIH 3T3 and other fibroblast cell lines have shown that pRb and C/EBPβ act together upstream of the peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα to convert fibroblasts into adipocytes (8, 15, 32, 72, 76–78). Likewise, it is clear that SF-1 is involved in a program of events that generates a differentiated phenotype, as $P450_{SCC}$ is inducible by cAMP and RA only in ES cells stably transfected with SF-1. These agents do not enhance SF-1 transcriptional activity, however, and require ongoing protein synthesis for activity, indicating that other, newly synthesized factors are required.

The ability of SF-1 to convert ES cells requires multiple domains, including that provided by the AF2 activation domain. AF2 consists of a hexameric polypeptide sequence that serves as the target for a number of interactive proteins whose ultimate purpose is to transduce activation function to the basal transcriptional machinery (for a review, see reference 31). AF2 furthermore permits ligand-induced relief from corepressors for some nuclear receptors (3, 11). It is therefore tempting to hypothesize that the role of AF2 in a nuclear receptor-mediated biological phenomenon is highly refined. Intriguingly, however, SF-1 mutants lacking AF2 (ΔAF2 or 1–277) can be rescued successfully by fusion to the viral activator VP16, indicating that for ES cell differentiation, SF-1 requires AF2 to achieve a threshold level of transcriptional activation, but no unique attribute is harbored within this region to transduce the signal. The interchangeability of transactivation domains in biological phenomena has also been noted in myogenic differentiation, whereby myogenic transcription factors with VP16 replacements of endogenous activation domains are still competent to drive differentiation (reviewed in reference 55). This feature has also been seen with the p53 activation domain and its ability to induce G_1 cell cycle arrest (1), as well as the glucocorticoid receptor's N-terminal (A/B) activation domain in the induction of thymocyte apoptosis (12). Our results demonstrate that a nuclear receptor can transduce a biological function with a heterologous acti-

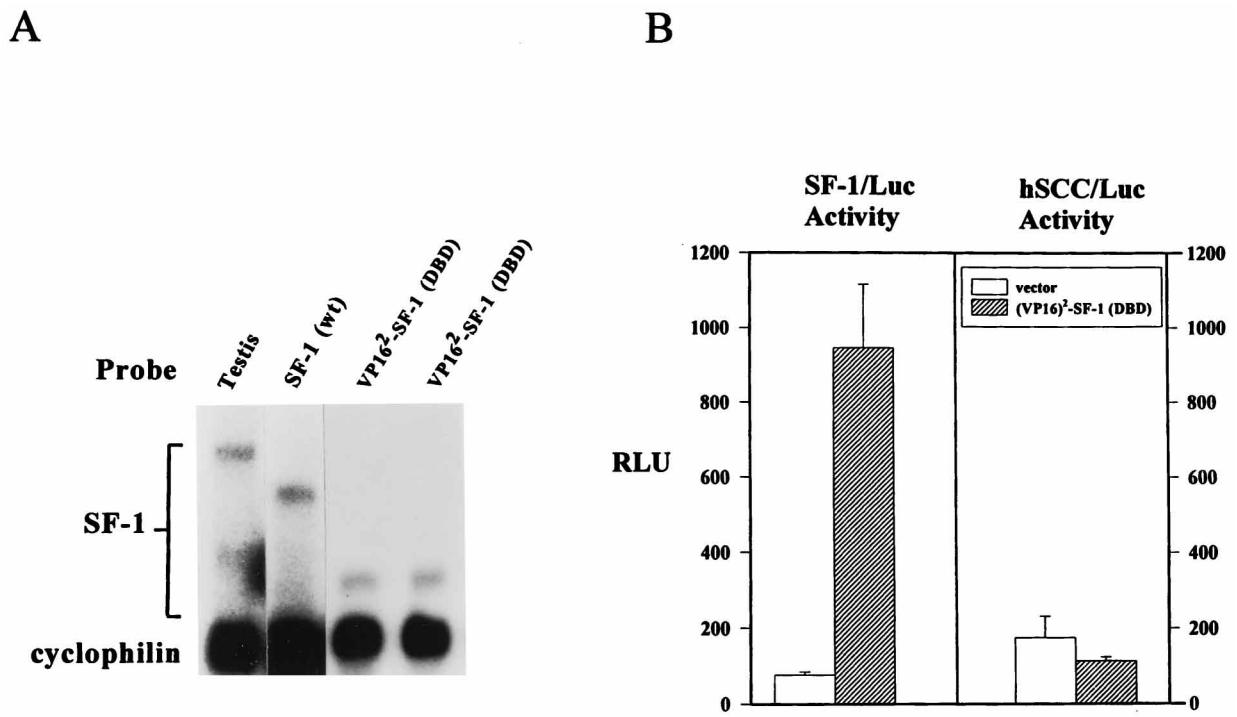

FIG. 7. The potent activator $VP16^2$-SF-1 (DBD) does not differentiate ES cells. (A) Northern analysis of SF-1 expression in testis, SF-1 (wt)-expressing ES cells, and two independent clones of $VP16^2$-SF-1 (DBD)-expressing ES cells. A ^{32}P-labeled SF-1 probe was generated and hybridized as described in Materials and Methods. Note three distinct mobilities conferred by the endogenous, full-length transgene and mutant transgene. While the full-length and mutant transgenes utilized the same untranslated vector-derived sequences, the length of the coding portion of the former is significantly longer than that of the latter. RNAs were equally loaded, as demonstrated by probing for cyclophilin. (B) Relative luciferase units (RLU) of reporters in native cells cotransfected transiently with empty vector or $VP16^2$-SF-1 (DBD) and SF-1-responsive reporters. Activities from SF-1/Luc and hSCC/Luc reporters were obtained and analyzed as described in the legend to Fig. 5.

vation domain replacing AF2, and it will be interesting to see if other receptors behave similarly.

SF-1 initiates a fate-determining program in ES cells through a process that remarkably does not require embryoid-body formation and proceeds under culture conditions that impede differentiation. However, the conversion is not a complete differentiation. For example, the cAMP-induced progesterone levels yielded by SF-1-converted ES cells are considerably lower than those produced by the Y1 adrenocortical cell line (data not shown). Given the high expression of $P450_{SCC}$ in steroidogenic ES cells, the deficiency may lie within cholesterol storage and mobilization pathways. Additionally, of the steroid hydroxylase genes and the steroidogenic acute regulatory protein, only $P450_{SCC}$ appears to be specifically induced in this phenomenon (data not shown). Finally, the converted ES cells were not responsive to ACTH or hCG (data not shown), which induce steroidogenesis in adrenocortical and gonadal cells, respectively, indicating that the membrane receptors for these hormones are probably not expressed by these cells. The differences between steroidogenic ES cells and steroidogenic tissue may arise because SF-1 is not expressed in vivo until E9 (35), after the urogenital mesoderm has been partitioned from pluripotent cellular lineages. This suggests that mesodermal cells may undergo early differentiation steps before SF-1 normally functions to drive differentiation into steroidogenic cells. Additionally, other transcription factors that act downstream of SF-1 are presumably required for terminal differentiation of cells that generate glucocorticoids, mineralocorticoids, or sex steroids by virtue of expression of more specific hydroxylases (58). Thus, while the undifferentiated stem cell is partially competent to respond to the signals transduced through SF-1 activity, SF-1 may require both upstream and downstream events to permit complete differentiation of ES cells.

Equally plausible is that terminal steroidogenic differentiation requires synergistic factors to act in concert with SF-1 to allow for physiologic degrees of activity on target promoters. This property has been noted in adipocyte differentiation with PPARγ, whose adipogenic activity is markedly increased by the presence of C/EBPα (72). Analogously, myogenic conversion of fibroblasts by basic helix-loop-helix transcription factors is dramatically enhanced by MADS domain transcription factors (54, 55). SF-1 is already known to cooperate with one transcription factor family in another system, as SF-1 and NGFI-A (Egr-1) collaborate in pituitary gonadotrophs to maximize expression of the β subunit of luteinizing hormone (46). Moreover, SF-1's closest mammalian homolog, LRH, requires cooperative activity from an unknown transcription factor to appropriately activate the alpha-fetoprotein gene in liver (24). Because our studies have unveiled a domain(s) between residues 105 and 277 within SF-1 that is (are) required for expression of $P450_{SCC}$ but not for transcriptional activation from an SF-1 reporter, the possibility that an interacting factor synergizes with SF-1 to generate the steroidogenic phenotype seems likely. As the retinoid X receptor (RXR) interacts with the monomeric orphan receptors of the NGFI-B family (22, 63) and RA stimulates steroidogenesis in SF-1-converted ES cells, perhaps an interaction between SF-1 and RXR would explain the phenotype of these cells. Transient transfection experiments have shown, however, that SF-1 and RXR do not functionally interact in the absence or presence of 9-*cis*-RA (data not shown). Another possible interactive transcription factor is DAX-1, a divergent orphan receptor which shares a similar expression pattern with SF-1 and whose mutation in humans gives phenotypes similar to that seen in SF-1 knockout mice (36, 39, 71, 80). Alternatively, SF-1 may be capable of using this region to cooperate with several tissue-specific factors, and the unique product of each of these interactions could contribute to the specification of the variety of subtypes of differentiated steroidogenic cells.

Finally, terminal differentiation of ES cells by SF-1 may be influenced by an unknown ligand. As lipophilic acid activators of PPARγ, and in particular, its ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, dramatically enhance the adipogenic effect of PPARγ (21, 43), SF-1 may have a ligand that dramatically enhances its activity. All of the possible interactive influences are not mutually exclusive; rather, it is plausible that the mechanism by which SF-1 orchestrates the differentiation of steroidogenic cells involves upstream gene activation steps, synergizing transcription factors, an inducing ligand, as well as downstream activation events.

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