Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10939–10943, November 1995
Developmental Biology

Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids

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Communicated by William H. Daughaday, Balboa Island, CA, August 14, 1995

ABSTRACT **The orphan nuclear receptor steroidogenic factor 1 (SF-1) is expressed in the adrenal cortex and gonads and regulates the expression of several P450 steroid hydroxylases *in vitro*. We examined the role of SF-1 in the adrenal glands and gonads *in vivo* by a targeted disruption of the mouse SF-1 gene. All SF-1-deficient mice died shortly after delivery. Their adrenal glands and gonads were absent, and persistent Mullerian structures were found in all genotypic males. While serum levels of corticosterone in SF-1-deficient mice were diminished, levels of adrenocorticotropic hormone (ACTH) were elevated, consistent with intact pituitary corticotrophs. Intrauterine survival of SF-1-deficient mice appeared normal, and they had normal serum level of corticosterone and ACTH, probably reflecting transplacental passage of maternal steroids. We tested whether SF-1 is required for P450 side-chain-cleavage enzyme (P450scc) expression in the placenta, which expresses both SF-1 and P450scc, and found that in contrast to its strong activation of the P450scc gene promoter *in vitro*, the absence of SF-1 had no effect on P450scc mRNA levels *in vivo*. Although the region targeted by our disruption is shared by SF-1 and by embryonal long terminal repeat-binding protein (ELP), a hypothesized alternatively spliced product, we believe that the observed phenotype reflects absent SF-1 alone, as PCR analysis failed to detect ELP transcripts in any mouse tissue, and sequences corresponding to ELP are not conserved across species. These results confirm that SF-1 is an important regulator of adrenal and gonadal development, but its regulation of steroid hydroxylase expression *in vivo* remains to be established.**

The steroid receptor family of transcription factors is involved in regulation of embryonic and postnatal tissue development, as well as differentiated functions. The orphan receptor steroidogenic factor 1 (SF-1) is highly expressed in steroidogenic tissues. It is expressed in all three layers of the adrenal cortex, in testicular Sertoli and Leydig cells, and in ovarian granulosa and theca cells (1). Like the orphan receptor NGFI-B, SF-1 binds as a monomer to a DNA element consisting of an estrogen receptor "half-site" (AGGTCA) and specific nucleotides immediately 5′ to the half-site (2). P450 side-chain-cleavage enzyme (P450scc), which catalyzes the initial and rate-limiting step in steroid biosynthesis, is encoded by a gene that contains half-site elements within its proximal promoter which are targets for SF-1 binding and transcriptional activation *in vitro* (3, 4). Similar SF-1 sites are found in genes encoding the steroid hydroxylases P450c17, P45011b, P450c21, and P450arom (3, 5, 6).

The amino acid sequence of SF-1 closely resembles that of Ftz-F1, an orphan nuclear receptor which may play a role in blastoderm and nervous system development in *Drosophila* (7, 8). Similarly, the expression of SF-1 as early as embryonic day 9 (E9) in the urogenital ridge, prosencephalus, and hypophyseal gonadotrophs suggests that SF-1 may also be an important regulator at multiple developmental stages (9, 10).

To study both the developmental and the differentiated functions of SF-1 *in vivo*, we generated mice which harbor a targeted disruption mutation in the zinc modules of SF-1, a domain which is indispensable for DNA binding. While we were analyzing the SF-1 −/− newborns, the phenotype of mice with a similar deficiency was described by Luo *et al.* (11), who reported that Ftz-F1 null mice lacked adrenal glands and gonads, which led to their early neonatal death. We report here a similar developmental defect in our SF-1 −/− mice and demonstrate that intact intrauterine survival can be explained by a normal antepartum level of corticosteroids. Furthermore, SF-1 is expressed in the placenta; however, normal levels of P450scc and P450c17 were found in placenta from SF-1 −/− mice, indicating that SF-1 is not required for their expression in this organ. Finally, SF-1 is thought to be derived from the same gene as embryonal long terminal repeat-binding protein (ELP), a putative transcription factor reported to be expressed in embryonal carcinoma cell lines (1, 12, 13). Because our mutation would also disrupt ELP function, we assessed the expression of ELP in steroidogenic tissues and embryonic cell lines, but no expression was detected, confirming that the observed phenotype is due to a deficiency in SF-1.

METHODS

Generation of SF-1-Deficient Mice. We have isolated a 14-kb genomic clone of the SF-1 gene from a 129Sv mouse genomic library and have replaced a 100-nt region between two *Pml* I sites within the first and second zinc finger of the DNA-binding domain with the PGK-neo cassette (Fig. 1*A*). This linearized construct was electroporated into the 129Sv embryonic stem (ES) cell line RW4. Homologous recombinants were selected with the neomycin analogue G418 and screened by Southern blotting. The targeted ES cells were then injected into C57BL/6 blastocysts and transferred into pseudopregnant female mice. Several SF-1 heterozygotes born to chimeric mice were mated to produce SF-1-deficient newborns. The genotype of each newborn mouse was analyzed for disrupted SF-1 alleles by Southern analysis.

Tissue Analysis. Newborn pups were examined within 12 hr after delivery. Embryos were obtained by abdominal delivery

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Abbreviations: ACTH, adrenocorticotropic hormone; ELP, embryonal long terminal repeat-binding protein; ES cell, embryonic stem cell; E*n*, embryonic day *n*; P450scc, P450 side-chain-cleavage enzyme; RT, reverse transcription; SF-1, steroidogenic factor 1.

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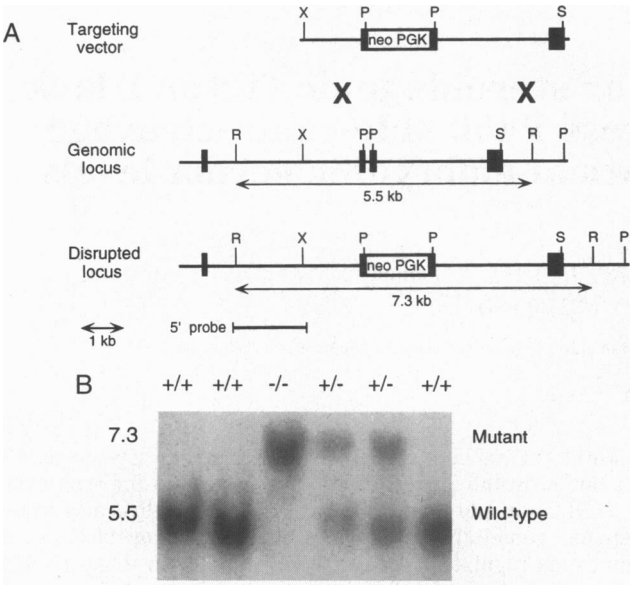

FIG. 1. Targeted disruption of the SF-1 gene. (*A*) Strategy used to target the SF-1 gene. The targeting vector contains the neomycin-resistance (neo) expression cassette, which includes a phosphoglycerate kinase (PGK) promoter and polyadenylylation signals. This cassette was inserted in the zinc modules of the DNA-binding domain of SF-1, thereby disrupting its DNA-binding capacity. Depicted restriction sites include *Pml* I (P), *Xho* I (X), *Sal* I (S), and *Eco*RI (R). Solid areas represent exons. The external probe used to distinguish wild-type (7.3-kb) and mutant (5.5-kb) alleles is shown. (*B*) Southern blot analysis of *Eco*RI-digested DNA samples from a litter resulting from mating of SF-1 heterozygotes, hybridized with the 5′ external probe. SF-1 −/− mutants are homozygous for the 7.3-kb allele.

of halothane-anesthetized pregnant mice at known gestational ages. Pups were sacrificed by decapitation and carefully examined. For histological evaluation, whole bodies or individual organs were fixed in 4% paraformaldehyde for 48 hr and embedded in paraffin. Seven-micrometer sections were cut and stained with hematoxylin and eosin.

Corticosterone and Adrenocorticotropic Hormone (ACTH) Assays. Immediately after decapitation, blood was collected into syringes containing EDTA, then plasma was separated by centrifugation and stored at −20°C. For corticosterone, the plasma was extracted twice with ether/ethyl acetate (1:1, vol/vol), and levels were measured by radioimmunoassay using a rabbit anti-corticosterone antibody (Sigma), according to the manufacturer's instructions. Pooled plasma ACTH levels were determined by an antibody sandwich assay using a chemiluminescence kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Corticosterone and ACTH interassay variation was 12% and 15%, and intraassay variation was 8% and 16%, respectively.

Cell Culture and Transient Transfections. F9 embryonal carcinoma cells were grown in NCTC 135 medium supplemented with 10% fetal bovine serum, 20% (wt/vol) glucose, 10 mM 2-mercaptoethanol, and antibiotics. ES cells were grown as described (14). CV-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Four million CV-1 cells at 50% confluence were transiently transfected by calcium phosphate coprecipitation (15), with 10 μg of expression vector containing the ELP cDNA downstream from a Rous sarcoma virus (RSV) promoter (gift from O. Niwa, Hiroshima University, Japan).

Expression Analysis. P450scc expression was determined by Northern blot hybridization analysis. For this analysis, the PolyATract system IV (Promega) was used to isolate poly(A)$^+$ RNA from total RNA, which was prepared as described (16). For determination of SF-1 expression, a reverse transcription (RT)–PCR assay was used. RNA samples were reverse transcribed and amplified with an SF-1-specific forward primer (5′-TGCACTGCAGCTGGACCGCCAGGAGTT-3′) and reverse primer (5′-AGGGCTCCTGGATCCCTAATGCAAGGA-3′). These primers are located on either side of a 2.5-kb intron and therefore do not produce a product from genomic DNA template. PCR was carried out for 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min.

To determine ELP expression, mRNA was isolated from tissues or cells, reverse transcribed, and amplified with a forward primer (5′-CAAACGAATCTGGATGGAAATGC-3′) and reverse primer (5′-GAAGCGGCAGAAGGGACAGCGG-3′). The product was electrophoresed in a 1.8% agarose gel, transferred to Sureblot hybridization membrane (Oncor), and hybridized to a labeled 240-nt ELP probe. The probe was generated by using the forward ELP primer described above and a reverse primer (5′-GCGTCCGCCTGTGGAAGGAGGAT-3′) for 40 cycles of 94°C, 1 min; 52°C, 1 min; 72°C, 1 min. The sexual genotype was determined by PCR analysis of the *sry* gene (11).

Human Genomic DNA Library Screening and Sequence Analysis. The human SF-1 gene was isolated from a λ Dash vector library (Stratagene), subcloned, and sequenced by the dideoxynucleotide method on an Applied Biosystems model 373A DNA sequencer. Nucleic acid and amino acid residue best-fit comparisons were carried out using the Lasergene sequence analysis program (DNAstar, Madison, WI).

RESULTS

SF-1-Deficient Mice Lack Adrenal Glands and Gonads. Using homologous recombination, we generated clones of ES cells which harbor a disruption mutation in the zinc finger module of one of their SF-1 alleles (Fig. 1). Two independent mouse lines, derived from two ES cell clones, yielded an identical phenotype. All mice heterozygous for the SF-1 gene disruption appeared healthy, grew and reproduced normally, and had a normal anatomy. A total of 106 embryos and newborns were examined. As demonstrated in Table 1, the ratio of genotypes indicates that the +/− or −/− genotypes do not increase embryonic lethality. Similarly, mean weights of +/+, +/−, and −/− newborn mice were not significantly different, suggesting that the disruption of SF-1 does not lead to intrauterine growth retardation. In contrast, we could not identify any live SF-1 −/− animals beyond 48 hr after birth, indicating a complete penetrance of the disrupted SF-1 phenotype.

A morphological comparison of the SF-1 −/− mice with that of wild-type mice revealed a total absence of the adrenal gland in all SF-1 −/− animals (Fig. 2 *A–C*), a finding that was confirmed by histological analysis (Fig. 2 *D–G*). Interestingly, even though SF-1 expression is limited to the cortex, the adrenal medulla was also absent. Immunohistochemistry with anti-tyrosine hydroxylase antibodies did not reveal any ectopically located clusters of sympatho-adrenal precursor cells outside the sympathetic nervous system (data not shown). These findings expand the role for SF-1 beyond the regulation

Table 1. Mice born from matings of SF-1 heterozygotes

Genotype	*n** (%)	% females	Birth weight, g
+/+	24 (23)	58	1.40
+/−	52 (49)	57	1.28
−/−	30 (28)	63	1.29
	$P = 0.83$†	$P = 0.87$†	$P = 0.24$‡

*Total = 106.
†Compared with expected ratios by χ^2 analysis.
‡One-way analysis of variance.

of steroidogenesis and indicate the dependence of adrenal gland development on SF-1 expression.

Because of the high gonadal expression of SF-1 in a normal mouse, we examined the sexual development of SF-1 −/− newborns. No gonads were detected in genotypically male or female mice (Fig. 2 *A–C*). The absence of adrenal glands and gonads was evident at E13 (data not shown), which is consistent with the expression of SF-1 in the urogenital ridge as early as E9 and with differentiation of urogenital ridge progenitors into adrenal cortex and gonads between E10 and E12 (9). These findings, therefore, indicate that SF-1 is important for differentiation of the urogenital ridge into steroidogenic organs. In addition, all genotypically male SF-1 −/− newborns (n = 59) exhibited fully developed uteri and oviducts, consistent with a lack of testes, a site of Mullerian inhibitory substance production.

Normal Antenatal, but Markedly Reduced Postnatal, Serum Levels of Corticosterone in SF-1 −/− Mice. To correlate the lack of adrenal glands in SF-1 −/− mice with endocrine function, we initially measured serum levels of corticosterone. A significantly lower level of corticosterone was found in SF-1 −/− neonates when compared with normal (+/+ or +/−) mice (Fig. 3*A*). To ensure that SF-1 disruption did not cause disregulation of ACTH responsiveness, we also assessed basal ACTH levels in these animals. ACTH levels were markedly higher in the SF-1 −/− pups than in their normal littermates, indicating that their pituitary corticotroph function was intact (Fig. 3*B*).

Prolonged intrauterine corticosteroid deficiency could lead to developmental abnormalities (17, 18). To assess the time period over which SF-1 null mice were exposed to a lower level of steroids, we compared corticosterone levels in serum from SF-1 null and wild-type E20 embryos (Fig. 3*A*). We found no difference in corticosterone levels between the groups. Moreover, plasma ACTH levels were not higher in E20 SF-1 null embryos than in normal embryos (Fig. 3*B*). These findings most likely reflect transplacental passage of maternally derived corticosteroids.

SF-1 Is Present in the Placenta but Is Not Essential for P450scc Expression. The placenta is the only major steroid-producing organ which develops in SF-1 −/− mice. Placental steroidogenesis takes place in trophoblast-derived cells (19–21). These cells originate in fetal tissues and express P450scc (19), a known target for SF-1 regulation *in vitro* (4). Using RT–PCR, we found that SF-1 is expressed in the normal placenta by E14 and thereafter. As expected, SF-1 was not expressed in placentas derived from SF-1 −/− mice (Fig. 4*A*). Placentas from SF-1 −/− mice were histologically normal (data not shown). Furthermore, we found similar levels of expression of P450scc mRNA in placentas derived from SF-1 +/+, +/−, and −/− mice (Fig. 4*B*). Thus, SF-1 is expressed in the placenta but is not required for placental development or for *in vivo* expression of P450scc. Expression of another steroid hydroxylase, P450c17, is regulated by SF-1 *in vitro* (6). Using RT–PCR, we detected P450c17 expression in placentas derived from SF-1 −/− embryos, and its level was indistinguishable from the level of P450c17 derived from SF-1 +/+ or +/− embryos (data not shown).

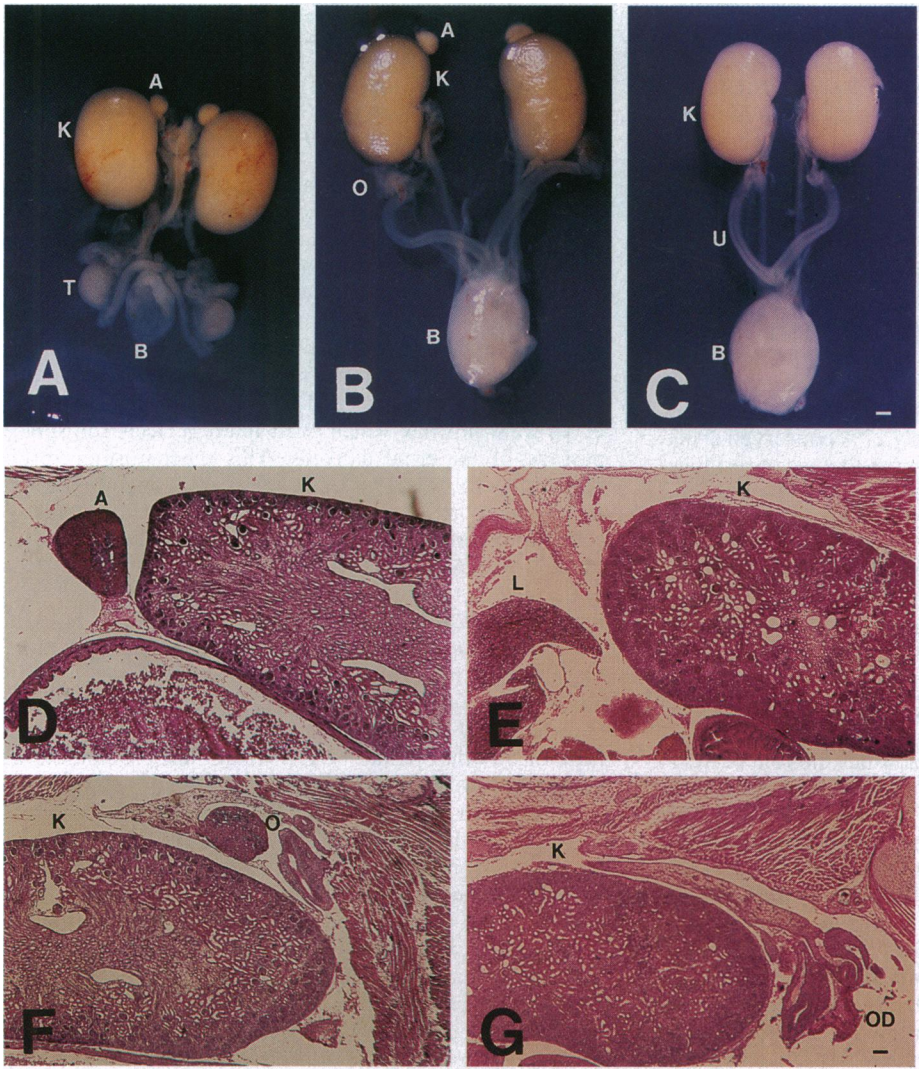

FIG. 2. Adrenal and gonadal phenotype of SF-1 −/− newborn mice. Macroscopic (*A–C*; bar = 500 μm) and microscopic (*D–G*; bar = 100 μm) analysis of representative animals are shown. Tissues were obtained as described in *Methods*, and microscopic sections were stained with hematoxylin and eosin. (*A*) Wild-type male. (*B*) Wild-type female. (*C*) SF-1 −/− male. (*D*) Wild-type male. (*E*) SF-1 −/− male. (*F*) Wild-type female. (*G*) SF-1 −/− female. Abbreviations: A, adrenal gland; B, bladder; K, kidney; L, liver; O, ovary; OD, oviduct; T, testis; U, uterus.

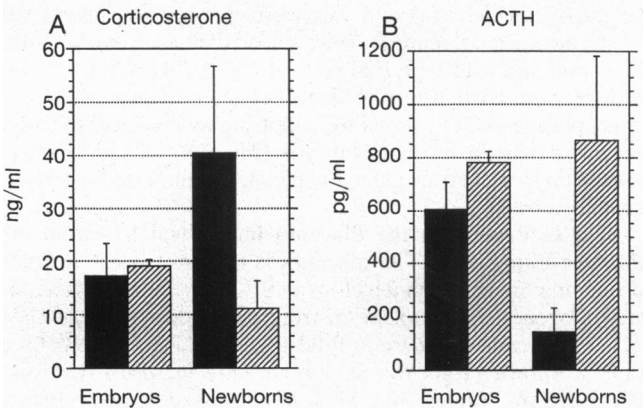

FIG. 3. Serum levels of corticosterone (*A*) and ACTH (*B*) in wild-type (solid bars) and SF-1 −/− (hatched bars) embryos and newborns. Corticosterone was measured by radioimmunoassay. ACTH was measured with an antibody sandwich assay. Results are expressed as mean and SD. (*A*) Corticosterone levels; $P = 0.68$ for embryos, and $P < 0.001$ for newborns. (*B*) ACTH levels; $P = 0.12$ for embryos, and $P < 0.005$ for newborns.

ELP Is Not Expressed in Steroidogenic Tissues and Is Not Conserved Across Species. We targeted the zinc finger module of SF-1 to create our null mutation, so it was possible that the phenotype we observed was due to a deficiency of SF-1 and/or of the alternatively spliced ELP. To determine the relevance of ELP expression to steroidogenic tissues, we attempted to detect ELP transcripts in RNA isolated from steroidogenic tissues of wild-type mice. We generated two primers for RT–PCR of RNA from those tissues (Fig. 5*A*). To separate the product from amplified genomic DNA, we designed the primers to span a short intron (145 nt) within the zinc module of the gene. The sensitivity of the PCR assay, determined with serial dilutions of ELP cDNA in RT mix, was <10 molecules of ELP per reaction. No 460-nt band corresponding to the size predicted for ELP mRNA was detected in any of the tissues or cell types (Fig. 5*B*).

To further investigate the potential existence of ELP, we examined the interspecies conservation of ELP coding sequences. We sequenced the region encoding the amino terminus of human ELP and compared it with the corresponding mouse sequence (Fig. 5*C*). While >90% interspecies homology was found for residues shared by SF-1 and ELP, putative coding regions unique to ELP were only 27% conserved. This low level of conservation was also observed between residues representing the translated intronic sequences (17%; see Fig. 5*C*), suggesting that the putative ELP-specific coding region does not encode a polypeptide. Similarly, mouse and bovine putative ELP-specific protein sequences were also only 18% conserved (data not shown). These findings provide strong support for the idea that the disruption of SF-1, and not ELP, underlies the abnormal phenotype observed in these mice.

DISCUSSION

The phenotype of SF-1 −/− mice establishes the role of SF-1 as an essential developmental regulator. These mice lack adrenal glands and gonads, resulting in a low serum level of corticosteroids and early neonatal death. Our results are in agreement with a recent report by Luo *et al.* (11), who found a similar phenotype in mice with an SF-1/ELP disruption. The function of SF-1 as a determinant of urogenital ridge morphogenesis is entirely consistent with its pattern of expression in embryonic tissues, as SF-1 appears in the urogenital ridge prior to differentiation into adrenal glands and gonads, which normally commences on E10 and continues throughout gestation (9, 11). In addition, SF-1 −/− newborn mice of both

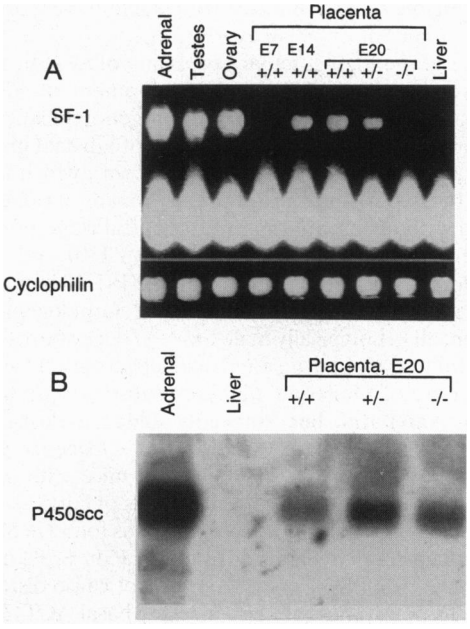

FIG. 4. SF-1 and P450scc expression in placenta. (*A*) RT–PCR analysis of SF-1 expression in placentas derived from embryos at indicated gestational ages. Adrenal gland and gonadal expression of SF-1 from wild-type mice is shown as control. Amplification was performed with SF-1-specific primers that generate a 381-nt product. A duplicate reaction using cyclophilin primers was used to control for integrity of the assay. SF-1 and cyclophilin were resolved in a 1.8% agarose gel. (*B*) Northern analysis of P450scc poly(A)$^+$ RNA levels in placentas derived from SF-1 +/+, +/−, and −/− E20 embryos. β-Actin transcripts were used to control RNA loading and transfer (data not shown). Quantitated by densitometry, the signals from SF-1 +/− and SF-1 −/− embryos were 1.4- and 1.1-fold higher than wild type.

sexes retained their Mullerian structures. This may be attributable to the absence of upregulation of Mullerian inhibitory substance by SF-1 (22) or may result from testicular agenesis.

Not surprisingly, serum corticosterone levels in SF-1 −/− newborns were significantly lower than in their wild-type littermates. In the absence of a feedback inhibition, ACTH levels in SF-1 −/− newborns were elevated, confirming that the function of their pituitary corticotrophs was intact (23). In contrast, corticosterone and ACTH levels in SF-1 −/− embryos were normal, which most likely reflects transplacental passage of maternal steroids. This mechanism is probably responsible for intact intrauterine survival of embryos that are deficient in corticotropin-releasing hormone (24), fetuses with congenital adrenal hypoplasia (25), or embryos with mutations in P450scc (26).

Because SF-1 is not expressed in wild-type adrenal medulla, its absence in SF-1 −/− mice may reflect a lack of inductive influences by corticosteroids (27, 28). The normal intrauterine plasma levels of corticosterone in SF-1 −/− mice suggest that adrenal medullary cell differentiation may depend on an elevated local concentration of corticosteroids, as suggested (29), or on other paracrine growth and differentiation factors elaborated by the adrenal cortex.

The known *in vitro* regulation of steroid hydroxylases by SF-1, along with its expression pattern *in vivo*, suggests a role for SF-1 in regulation of steroidogenesis (1–6, 30). The absence of adrenal glands and gonads in SF-1 −/− mice precludes the study of the role of SF-1 in regulating steroid hydroxylase expression in the major steroidogenic tissues, except the placenta. Our findings that normal placenta expresses SF-1 and that placentas from SF-1 −/− mice express both P450scc and P450c17 indicate that SF-1 is not required for their expression in this organ. Thus, we do not know whether

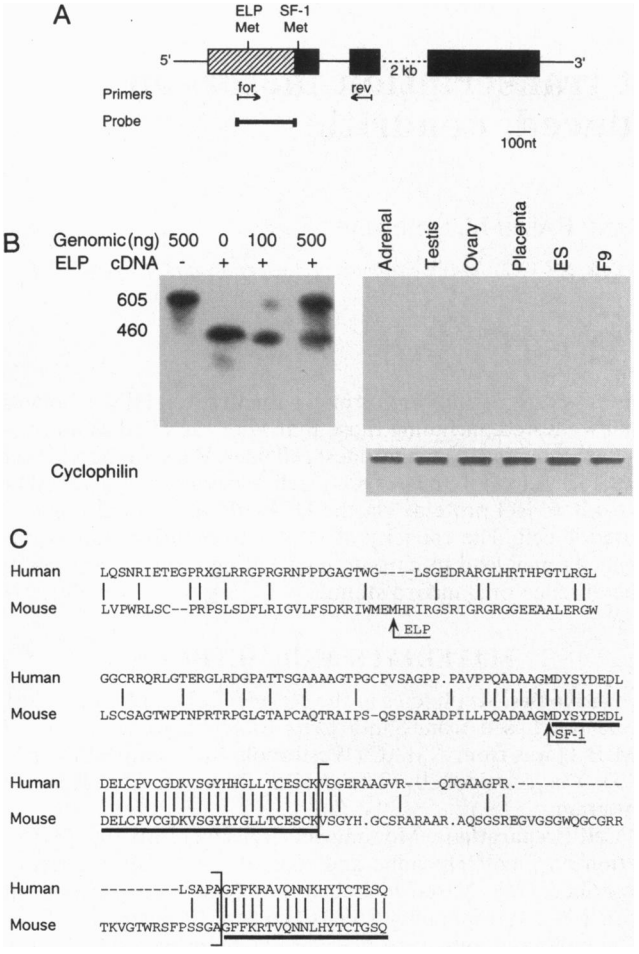

FIG. 5. ELP sequence analysis and expression in steroidogenic tissues and cell lines. (*A*) Strategy employed for detection of ELP transcripts. Hatched bar represents ELP-specific sequence; solid bars represent sequences shared by SF-1 and ELP. Forward (for) and reverse (rev) primers were designed to detect transcripts specific to the amino terminus of ELP. These primers span a 145-nt intron located within the zinc module of SF-1/ELP and therefore distinguish an ELP-specific product (460 nt) from fragments amplified from genomic DNA (605 nt). The ELP-specific probe used for Southern blotting is shown. (*B*) ELP transcripts cannot be detected by RT–PCR analysis. Amplifications of genomic DNA and RNA extracted from CV-1 cells transfected with an ELP expression vector are shown to demonstrate the product sizes generated from the ELP/SF-1 genomic loci and ELP mRNA, respectively. A Southern blot with a ^{32}P-labeled ELP-specific probe was used to enhance detection of the PCR product. (*C*) Comparison of translated human and mouse SF-1 sequences. Analysis was performed for all three reading frames, and the best-fit frame is presented. Vertical lines show identical amino acids. Dashes indicate gaps introduced to achieve optimal alignment. The methionine residues corresponding to SF-1 and ELP translation start sites are marked. Amino acid residues corresponding to coding sequences are underlined. The intron within the SF-1/ELP DNA binding domain is included for comparison and is delimited by brackets.

SF-1 is necessary for expression of steroid hydroxylases in the adrenal glands or gonads *in vivo*.

The existence of the transcriptional repressor ELP, which shares a high degree of homology with FTZ-F1 and SF-1, has been previously proposed (12, 13, 31). ELP is identical to SF-1 within the DNA-binding domain but differs from SF-1 by possessing a longer amino terminus and a shorter carboxyl terminus (12). Because the region of the gene disrupted in our targeting construct is shared by SF-1 and ELP, it is plausible that the phenotype we observed may reflect the absence of both genes. However, our results indicate that ELP expression is irrelevant to our system. First, in most reports ELP was not found in any embryonic tissue (1, 11). Using intron-spanning ELP primers in a RT–PCR assay, we did not detect ELP expression in the adrenal glands, gonads, placenta, or in any other cells tested. Second, we analyzed protein sequences from mouse, human, and bovine ELP and found a low degree of homology across the putative coding sequences tested. In contrast, regions shared by SF-1 and ELP were almost identical. Interestingly, Nomura *et al.* (32) have also found a functionally significant sequence divergence between rat and mouse ELP. Taken together, these results support our conclusion that the observed phenotype of the SF-1 −/− mouse reflects a disruption in SF-1 alone, since it is questionable whether ELP exists.

We thank Galina Gavrilina and Robin Wesselschmidt for technical assistance in the generation of SF-1 −/− mice and preparation of histological slides, Pam Goda for animal husbandry, and Robert Heuckeroth and Brad Sevetson for their critical comments on the manuscript. This work was supported by Grant P01 CA49712-06A1 from the National Cancer Institute. J.M. is an Established Investigator of the American Heart Association.

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