Developmental Expression of Mouse Steroidogenic Factor-1, an Essential Regulator of the Steroid Hydroxylases

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As an initial step toward understanding its role in steroidogenesis, we studied the developmental profile of steroidogenic factor-1 (SF-1), a nuclear receptor that regulates the steroid hydroxylases. SF-1 transcripts first appear on embryonic day 9 (E9) in the urogenital ridge, the probable source of steroidogenic cells of both adrenals and gonads. By E11, after the adrenals and gonads are clearly separate, SF-1 transcripts are detected throughout the adrenal primordium. Thereafter, adrenal expression of SF-1 localizes to the cortex. Consistent with its proposed role in regulating cholesterol side-chain cleavage enzyme (SCC), SF-1 is expressed before SCC.

During the sexually undifferentiated stage of gonadal development (E9–E12), all embryos express SF-1 in the genital ridge. As testicular cords form in males, SF-1 transcripts are diffusely expressed throughout the testis, whereas SCC mRNA is limited to the interstitium. These differences between SF-1 and SCC reflect SF-1 expression by Sertoli cells, as shown by Northern blotting and *in situ* hybridization. In contrast to its persistent expression in the embryonic testis, SF-1 transcripts disappear from the ovary between E13.5–E16.5, reappearing only during late gestation (E18.5). Thus, expression of SF-1 in the embryonic gonad is sexually dimorphic. Coupled with the demonstration of SF-1 mRNA in Sertoli cells, these data suggest that SF-1 plays a role in gonadal development distinct from regulating the steroidogenic enzymes. Additionally, SF-1 is expressed in the embryonic forebrain, implying a role in neural development. (Molecular Endocrinology 8: 654–662, 1994)

INTRODUCTION

The biosynthesis of steroid hormones requires the sequential action of a related group of cytochrome P450 steroid hydroxylases that convert cholesterol to the various steroid classes (reviewed in Ref. 1). We recently isolated and characterized an orphan nuclear receptor that interacts with shared promoter elements up-stream of the mouse steroid hydroxylases to regulate their expression (2). This protein, designated steroidogenic factor-1 (SF-1), is one of two transcripts encoded by the mouse homolog of *fushi tarazu* factor-1 (*FTZ-F1*) (3, 4), a *Drosophila* gene proposed to regulate the expression of the *fushi tarazu* (*ftz*) homeobox gene (5). In *Drosophila*, *FTZ-F1* also encodes two transcripts whose expression is temporally regulated: an early form that correlates with *ftz* expression and a late form of unknown function (6, 7).

We previously used *in situ* hybridization to show that SF-1 is expressed at early stages of adrenocortical development, with transcripts detected in the adrenal analage on embryonic day 12 (E12) (4). *In situ* hybridization analyses of adult mouse tissues revealed SF-1 expression in cortical cells of the adrenal gland, Leydig cells of the testis, and thecal and granulosa cells of the ovary, all of which are sites of steroidogenesis (4). As the next step in defining the role of this nuclear receptor in endocrine development and function, we now present a detailed analysis of SF-1 expression in the adrenal gland and gonads during postimplantation embryonic development. In addition, we compare its developmental profile in these tissues with that of cholesterol side-chain cleavage enzyme (SCC),[1] a gene that catalyzes the initial and rate-limiting step in the biosynthesis of steroid hormones. Finally, based on the finding that the rat homolog of SF-1, designated adrenal-4-binding protein, is expressed in brain RNA (8), we analyze SF-1 expression in the embryonic mouse brain. Our results are consistent with the idea that SF-1 plays a pivotal role in the function of steroidogenic tissues and impli-

[1] A standard nomenclature for the cytochrome P450 genes has recently been adapted (28). According to this system, the formal name for the gene encoding the cholesterol side-chain cleavage enzyme is *Cyp11A*.

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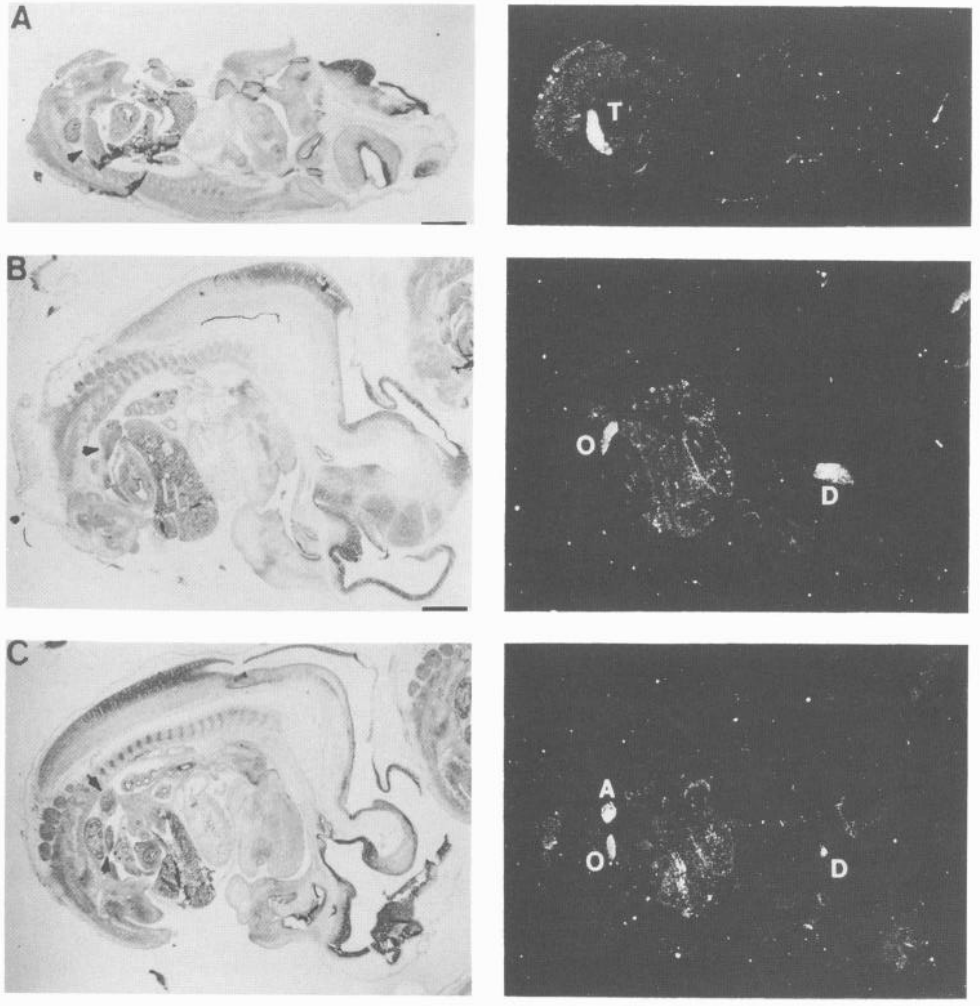

Fig. 1. SF-1 Expression in the Mouse Embryo Is Restricted to Primary Steroidogenic Tissues and the Diencephalon
Serial sagittal sections from mouse embryos at different stages were prepared and analyzed by *in situ* hybridization with an SF-1-specific cRNA probe, as described in *Materials and Methods*. A, E12.5 male embryo; brightfield and darkfield analyses with the SF-1 probe. B, E12.5 female embryo; brightfield and darkfield analyses with the SF-1 probe. C, More medial serial section from the same embryo used in B; brightfield and darkfield analyses with the SF-1 probe. T, Testis; A, adrenal; O, ovary; D, diencephalon. *Arrowheads* in the brightfield photographs indicate the sites of SF-1 expression. *Scale bars* = 500 μm.

cate SF-1 in gonadal development and the formation of a region of the developing hypothalamus.

RESULTS

SF-1 Is Selectively Expressed in Embryonic Adrenal Glands, Gonads, and Diencephalon

To assess SF-1 expression during embryogenesis, we first used *in situ* hybridization with sagittal sections of mouse embryos obtained at various stages of embryonic development. These studies revealed that SF-1 expression is highly restricted during development. On E12.5, as shown in Fig. 1, SF-1 transcripts are limited to the gonad, the adrenal primordium, and the diencephalon. In serial sections encompassing the rest of the embryo, no signal was detected (data not shown). The expression of SF-1 in the developing adrenal gland and gonads is in keeping with its restriction to steroidogenic cell types in the adult mouse (4).

SF-1 Is Expressed at the Earliest Stages of Adrenal Development

The steroidogenic components of both adrenal glands and gonads derive from coelomic epithelium located in the dorsal mesentery of the hind gut, termed the urogenital ridge. We wanted to determine when cells in this region first expressed SF-1. As shown in Fig. 2A, SF-1 transcripts are detected at the earliest stage of development of the urogenital ridge (E9), before the gonads and adrenal gland can be distinguished. Histological resolution of adrenals and gonads is not possible at this stage of development; however, careful analyses of serial sections at slightly later stages (E9.5–E10.5)

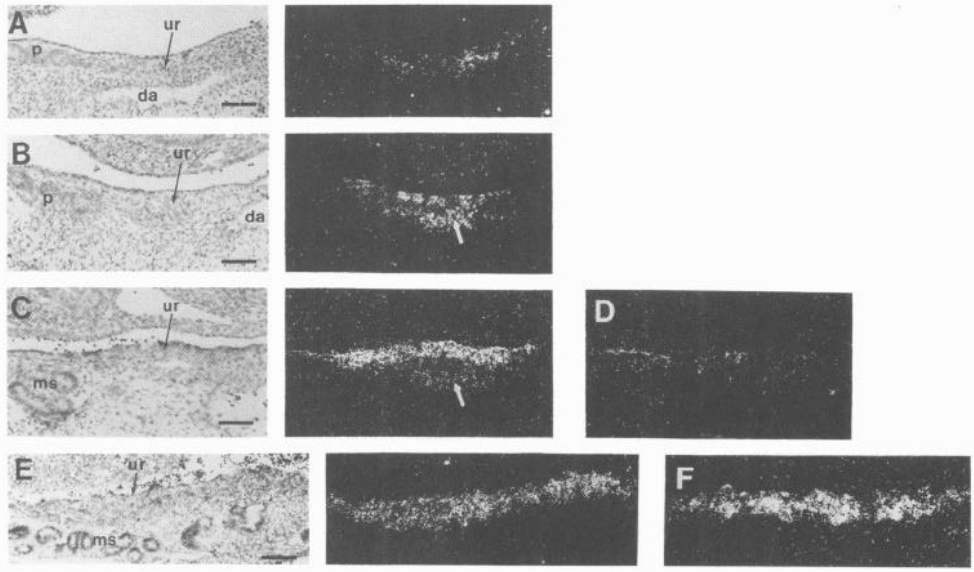

Fig. 2. SF-1 mRNA Is Expressed at the Earliest Stages of Urogenital Differentiation

Serial sagittal sections from mouse embryos at different stages were prepared and analyzed by *in situ* hybridization, as described in *Materials and Methods*. The probes included SF-1- and SCC-specific cRNA probes. A, E9.0; brightfield and darkfield analyses with the SF-1 probe. B, E9.5; brightfield and darkfield analyses with the SF-1 probe. C, E10.5; brightfield and darkfield analyses with the SF-1 probe. D, E10.5; darkfield analysis of the same embryo used in C with the SCC probe. E, E11.0, brightfield and darkfield analyses with the SF-1 probe. F, E11.0, darkfield analysis with the SCC probe. The *white arrows* in B and C indicate hybridization in regions dorsal and medial to the presumed urogenital ridge. *Scale bar* = 100 μm. ur, Urogenital ridge; p, pronephros; da, dorsal aorta; ms, mesonephros.

suggest that discrete populations of gonadal and adrenal cells are already forming, because some discontinuity in the SF-1 signal can be detected (see *white arrows* indicating SF-1 expression in the presumed adrenal primordium in Fig. 2, B and C). By E11–E12, SF-1 transcripts are clearly detectable in the adrenal primordium (Fig. 3, A and C), which by E12 can be distinguished from surrounding splanchnic mesoderm as a local collection of cells dorsal and medial to the mesonephros and gonads (Fig. 3C). Scattered within the cortical cells are neural crest-derived sympathetic cells that will eventually form the adrenal medulla. The adrenal gland becomes encapsulated on approximately E14–E14.5 (Fig. 3D) and continues to increase in size. By E16–E16.5 (Fig. 3E), the adrenal gland contains distinct cortical and medullary compartments, which represent the sites of biosynthesis of steroids and catecholamines, respectively. Throughout the remainder of gestation (Fig. 3E), SF-1 is expressed by the outer cortical region, where steroidogenic cells are found.

Serial sections from mouse embryos at the same stage were also hybridized with the SCC probe to examine the temporal relationship of SF-1 and SCC expression during adrenal development. As shown in Fig. 2D, SCC expression was not detectable on E10.5, at least 1 day after SF-1 expression in the presumed adrenal primordium was clearly detectable, and was first found in both gonad (Fig. 2F) and adrenal primordium (Fig. 3B) on E11, a time roughly comparable to the initiation of SCC expression in the developing rat embryo (9). The finding that SF-1 expression antedates that of SCC in the developing adrenal gland is consistent with the model that SF-1 is an obligatory regulator of SCC expression (10).

SF-1 and SCC Are Expressed in the Indifferent Gonad of Both Sexes

As described above, the urogenital ridges become visible on E9 on either side of the dorsal mesentery of the hind gut (Fig. 2A). On E11 (Fig. 2E), the gonadal primordium becomes discernible, although developing male and female embryos still cannot be distinguished morphologically; this stage is, therefore, termed the indifferent, or bipotential, gonad. By E11.5, primordial germ cells become visible within the gonadal blastema. By E12, the gonad has elongated, although it is still not possible to differentiate male and female embryos. On E12.5 (Figs. 4A and 5A), it first becomes possible to distinguish ovary from testis. The testis has a striped appearance because of the development of testicular cords, which will later become the seminiferous tubules (Fig. 4A). The ovary, in contrast, retains a homogeneous granular appearance (Fig. 5A). As development proceeds (Figs. 4C and 5C), the histological differences between ovary and testis are more pronounced. The testis enlarges, and the testicular cords become seminiferous tubules containing cords of tissue with embedded germ cells surrounded by interstitial tissue (Fig.

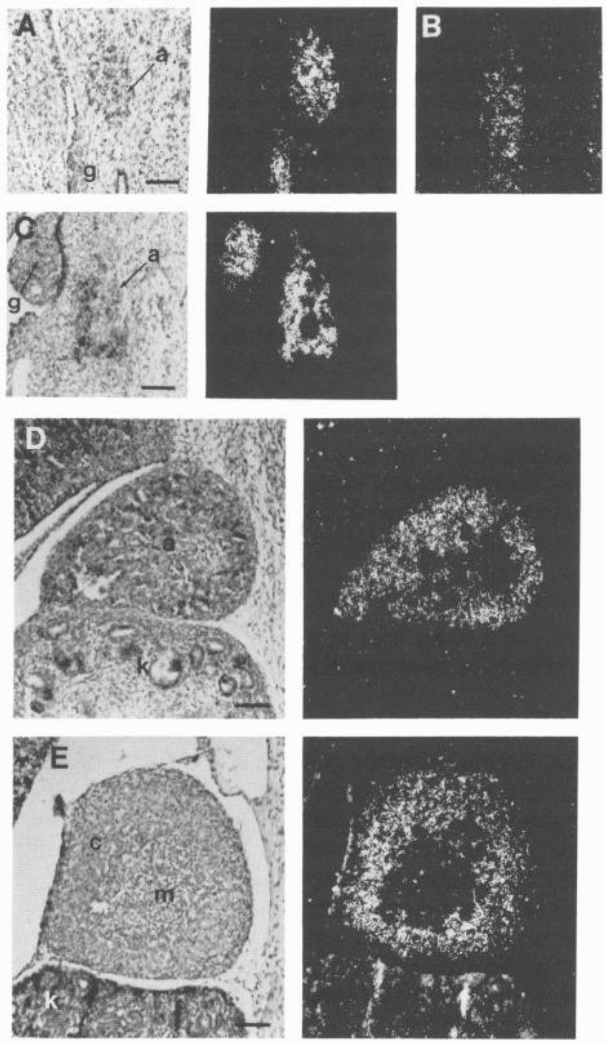

Fig. 3. Expression of SF-1 and SCC mRNAs in the Developing Adrenal Gland

Serial sagittal sections from mouse embryos at different developmental stages were prepared and analyzed by *in situ* hybridization, as described in *Materials and Methods*. A, E11.0; brightfield and darkfield analyses with the SF-1 probe. B, E11.0; darkfield analysis with the SCC probe. C, E12.0; brightfield and darkfield analyses with the SF-1 probe. D, E14.5; brightfield and darkfield analyses with the SF-1 probe. E, E16.5; brightfield and darkfield analyses with the SF-1 probe. *Scale bars* = 100 μm. a, Adrenal primordium; g, indifferent gonad; k, kidney; c, adrenal cortex; m, adrenal medulla.

4, C and D), whereas the ovary remains relatively undifferentiated (Fig. 5C) (11).

We were surprised to find that the indifferent gonads of all embryos on E9–E12 express SF-1 at levels that are readily detected (Fig. 2, B, C, and E). As noted above, it is impossible at the earliest stages (E9–E10.5) to differentiate with certainty the adrenal and gonads at sites of SF-1 expression, but we see subtle differences in the signal intensity when serial sections are examined (data not shown), suggesting that both adrenals and gonads express SF-1. The *arrows* in Fig. 2, B and C, illustrate examples of these regions of presumed adrenal signal.

All 20 embryos from stages E9–E12 that we examined by *in situ* hybridization express SF-1 in the genital ridge. This number of positive embryos strongly implies that both males and females express SF-1 at the initial stages of gonadal development. Further evidence for this came from polymerase chain reaction (PCR) amplification of genomic DNA from the mouse embryos with primers specific for the *Sry* gene, which is located on the Y-chromosome and plays a crucial role in sex determination (12). The absence of *Sry*-related products with a subset of the samples, at least five of which were verified to contain DNA adequate for PCR by control reactions with SF-1 primers, verifies that the embryos analyzed included females (data not shown). These results establish that SF-1 is universally expressed in the bipotential gonad, suggesting that it may play an important role in the earliest stages of gonadal development.

Sexually Dimorphic Expression of SF-1 Accompanies Gonadal Differentiation

As the gonads develop such that male and female embryos can be distinguished, sexually dimorphic expression of SF-1 becomes apparent. From E12.5–E15, the testis differentiates into distinct seminiferous tubules and interstitium. SF-1 is expressed at high levels in the testis at this stage. Although the highest levels of expression are found in the developing interstitial region, it is clear that testicular cords and seminiferous tubules also express SF-1 mRNA (Fig. 4, A, C, and D). Finally, by E18.5, SF-1 expression has diminished (Fig. 4E), perhaps reflecting the relative inactivity of Leydig cells in steroidogenesis at this stage of development (11).

Only a subset of testicular cells expressing SF-1 also express SCC. As shown in Fig. 4B, SCC expression in the testis is apparent on E12.5. In marked contrast to the expression of SF-1 in both interstitium and seminiferous tubules (Fig. 4A), SCC expression is strictly limited to the interstitial steroidogenic region (Fig. 4B). These differences in sites of expression suggest that SF-1, in addition to its function in the steroidogenic compartment of the testis, may contribute to the developing seminiferous tubule.

The expression of SF-1 in the developing ovary shows a very different pattern. As shown above, SF-1 was expressed in indifferent gonads of female embryos at levels comparable to those seen in males (Fig. 2E). On E12.5, as the ovary became morphologically distinct from the testis, the SF-1 signal was still seen (Fig. 5A). By this time, the SCC mRNA was no longer detected in the ovary (Fig. 5B). From E13.5–E16.5, neither SF-1 (Fig. 5, C and D) nor SCC was expressed in the developing ovary. Finally, on E18.5, a faint signal of SF-1 expression could be detected (Fig. 5E). This SF-1 expression presumably reflects the maturation of the ovary on E18–E18.5 to the primary follicle stage, char-

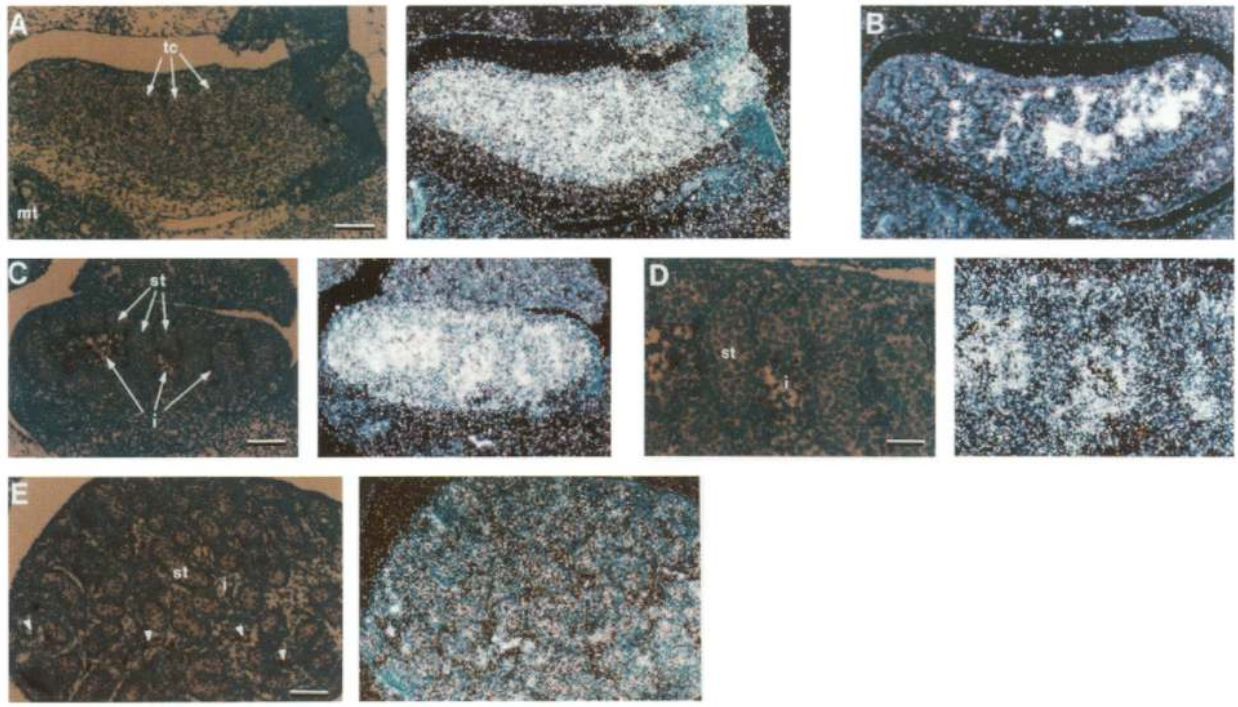

Fig. 4. Expression of SF-1 and SCC mRNAs in the Embryonic Testis

Serial sagittal sections from mouse embryos at different developmental stages were prepared and analyzed by *in situ* hybridization, as described in *Materials and Methods*. A, E12.5 testis; brightfield and darkfield analyses with the SF-1 probe. B, E12.5 testis; darkfield analysis of the same embryo used in A with the SCC probe. C, E13.5 testis; brightfield and darkfield analyses with the SF-1 probe. D. E13.5 testis, brightfield and darkfield analyses at higher power with the SF-1 probe. E, E18.5 testis; brightfield and darkfield analyses with the SF-1 probe. *Scale bars* = 100 μm, except in D, where it = 50 μm. tc, Testicular cord; mt, metanephros; st, seminiferous tubule; i, interstitial region. *Arrowheads* indicate blood vessels, which give artifactual signals.

acterized by primary oocytes at the dictyate stage of the first meiotic division surrounded by follicular cells (11).

Sertoli Cells on Postnatal Day 15 Express SF-1

The *in situ* hybridization studies described above strongly suggest that SF-1 is expressed in the seminiferous tubule, which consists of Sertoli and germ cells. We, therefore, analyzed the expression of SF-1 in a highly enriched population of Sertoli cells obtained from postnatal day 15 (P15) rats, a stage when testicular maturation is ongoing. As shown in Fig. 6, SF-1 mRNA is expressed in this cell population. Although Sertoli cells do not represent 100% of this population, as small numbers of peritubular cells are included in the sample, the cell population does not contain significant numbers of Leydig cells, as no signal was seen with a probe for 3β-hydroxysteroid dehydrogenase (Fig. 6). These results, thus, suggest that Sertoli cells are the site of SF-1 expression.

Additional evidence that Sertoli cells express SF-1 came from *in situ* hybridization analysis of the highly enriched primary Sertoli cells with the mouse SF-1 probe. As shown in Fig. 7, hybridization was seen in most of the Sertoli cells, directly documenting Sertoli cells as sites of SF-1 expression. The localization of SF-1 to Sertoli cells thus implies that SF-1 contributes to the embryonic differentiation of both compartments of the fetal testis.

SF-1 mRNA Is Expressed in the Developing Prosencephalon

Previous reports of SF-1 expression in the rat brain (8) prompted us to examine SF-1 expression in the embryonic brain by *in situ* hybridization. As shown in Fig. 8A, a prominent signal was visible in E11.5 embryos, limited to the rostral part of the basal plate of the secondary prosencephalon. This region of the developing forebrain contains primordia of the retrochiasmatic and tuberal hypothalamus. By E14.5, the region in which SF-1 was expressed was more clearly associated with the developing hypothalamus (Fig. 8B). Other regions of the developing brain did not exhibit SF-1 expression (data not shown). In additional experiments with coronal sections and in analyses of serial sections containing other regions of the developing brain, SF-1 expression was again strictly limited to the retrochiasmatic region, which includes hypothalamic primordia (data not shown).

DISCUSSION

Recent studies have implicated an orphan nuclear receptor, alternatively designated SF-1 or adrenal-4-

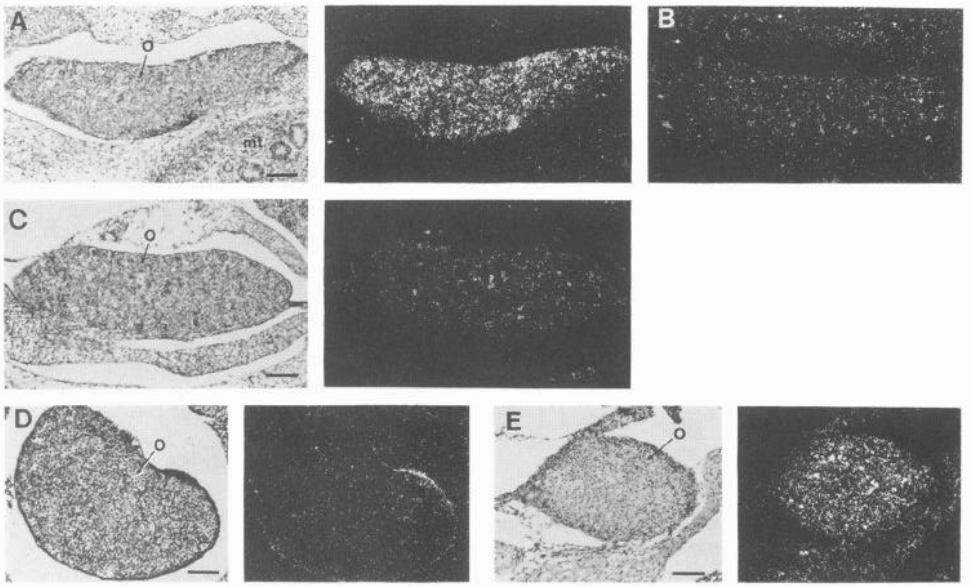

Fig. 5. Expression of SF-1 and SCC mRNAs in the Embryonic Ovary

Serial sagittal sections from mouse embryos at different developmental stages were prepared and analyzed by *in situ* hybridization, as described in *Materials and Methods*. A, E12.5 ovary; brightfield and darkfield analyses with the SF-1 probe. B, E12.5 ovary; darkfield analysis of the same embryo used in A with the SCC probe. C, E14.5 ovary; brightfield and darkfield analyses with the SF-1 probe. D, E16.5 ovary; brightfield and darkfield analyses with the SF-1 probe. E, E18.5 ovary; brightfield and darkfield analyses with the SF-1 probe. *Scale bars* = 100 μm. o, Ovary; mt, metanephros.

binding protein in gene regulation of the cytochrome P450 steroid hydroxylases (2, 4, 8, 10). In this report, we examine the temporal relationship during embryonic development between SF-1 and SCC, one of these steroid hydroxylases. Results show that SF-1 is expressed at the earliest stages of urogenital ridge development, preceding the expression of SCC. In the developing ovary, cessation of SF-1 expression accompanied the loss of SCC expression. Although we did not examine the developmental profiles of SF-1 and SCC protein, a recent report in the rat showed good agreement between *in situ* hybridization and immunohistochemical determination of the onset of SCC expression (9). Certainly, the relative expressions of SF-1 and SCC mRNAs are consistent with the model that SF-1 is necessary, if not sufficient, for SCC expression.

Sexual differentiation requires the interaction of a number of genes that must be activated at the appropriate stages to allow sexually dimorphic development. Until day E12.5, male and female mouse embryos are histologically indistinguishable. Recent studies have defined a gene, called *Sry*, that maps to the minimum sex-determining region of the Y-chromosome and is expressed within a developmental window (E10.5–E12.5) consistent with a role in testicular development (12). Although the precise mechanisms remain to be elucidated, *Sry* is believed to encode the testis determining factor, whose expression most likely initiates a cascade of down-stream events that lead to testicular differentiation and assumption of the male phenotype.

A second requirement for male sexual differentiation *in utero* is the production of androgens. After its biosynthesis by the fetal testis, testosterone interacts with the androgen receptor to direct programmed developmental changes in the external genital system. Testosterone production requires the sequential actions of two cytochrome P450 steroid hydroxylases: cholesterol side-chain cleavage enzyme and steroid 17α-hydroxylase. Previous studies indicate that SF-1 regulates SCC expression in adrenocortical cells (13) and primary cultures of granulosa cells (14).

To the extent that mRNA levels of SF-1 and SCC correlate with protein levels, the detection of SF-1 before SCC is consistent with the model that SF-1 regulates steroid hydroxylase gene expression. The results presented here further implicate SF-1 in pathways that extend beyond its role in regulating the steroid hydroxylases. Expression of SF-1 in the indifferent gonads of male and female embryos at a time when steroid production is not needed for female sexual development implies a broader role in gonadal differentiation. The onset of SF-1 expression probably precedes that of *Sry*, which was first detected by *in situ* hybridization on E10.5 (12). A subsequent study, however, suggests that *Sry* expression can be detected by more sensitive reverse transcription-PCR techniques in the preimplantation mouse embryo (15), so the precise temporal relationship of these two genes remains unresolved and merits further study. It will, likewise, be very interesting to compare directly the temporal relationship between SF-1 and the Wilm's tumor-associated gene, which is also detected at the earliest stages of organogenesis in the urogenital ridge (16).

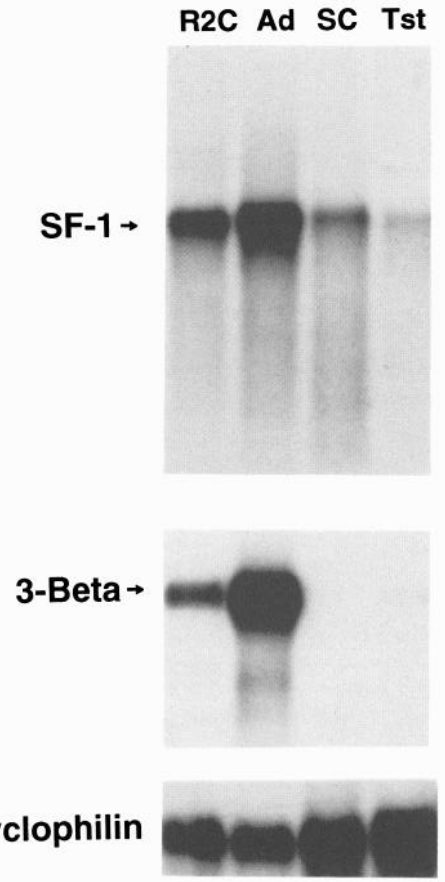

Fig. 6. Detection of SF-1 Transcripts in Sertoli Cells by RNA Blot Analysis

Highly enriched Sertoli cells (SC) from P15 testes were prepared and cultured, and total RNA was prepared, as described in *Materials and Methods*. Total RNA was also prepared from rat R2C Leydig tumor cells, whole rat adrenal glands (Ad), and whole rat testes (Tst). The samples were analyzed by Northern blotting analysis. The probes used for hybridization included rat SF-1 (19), a probe for rat 3β-hydroxysteroid dehydrogenase that is specific for Leydig and adrenocortical cells, and a control probe for cyclophilin.

Our observations that mRNA from highly enriched Sertoli cells contains SF-1 transcripts and that Sertoli cells are positive for SF-1 mRNA by *in situ* hybridization assays (Figs. 6 and 7) implicate SF-1 in a role beyond the regulation of steroid hydroxylases, as SCC is strictly confined to the interstitium (Fig. 4B). These findings mandate a reevaluation of the role of SF-1 in gonadal differentiation and function. There are several potential roles for SF-1 in Sertoli cell function. Although SCC is not expressed in the seminiferous tubules, prepubertal Sertoli cells express aromatase, the cytochrome P450 steroidogenic enzyme that converts androgens to estrogens (17, 18). Given the recent demonstration that SF-1 regulates aromatase expression in gonadal cells (19, 20), SF-1 expression in prepubertal Sertoli cells may be related to that of aromatase. Alternatively, SF-1 may regulate other, as yet unidentified, genes that are required for seminiferous tubule function.

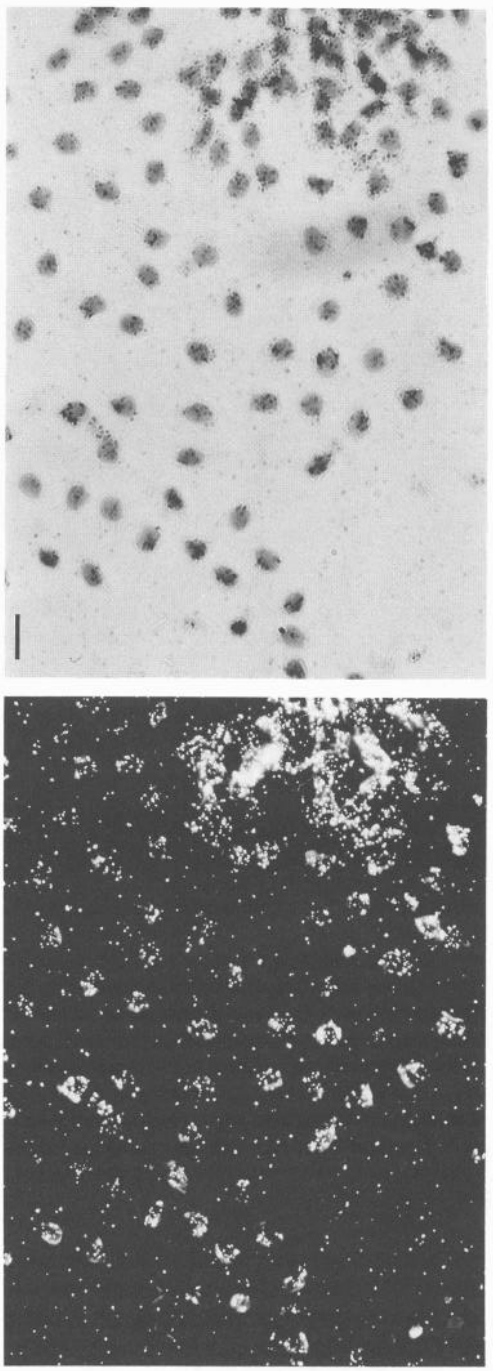

Fig. 7. Detection of SF-1 Transcripts in Primary Rat Sertoli Cells by *in Situ* Hybridization

Highly enriched Sertoli cells were prepared from P15 testis and analyzed by *in situ* hybridization, as described in *Materials and Methods*. The pictures are bright- and darkfield views at ×40 magnification. *Scale bar* = 20 μm.

With respect to the potential broader role of SF-1, its expression within a discrete region of the developing prosencephalon is also of considerable interest. Although the underlying mechanisms remain to be defined, it is intriguing that SF-1 is expressed within a region of the hypothalamic primordia, thus implicating

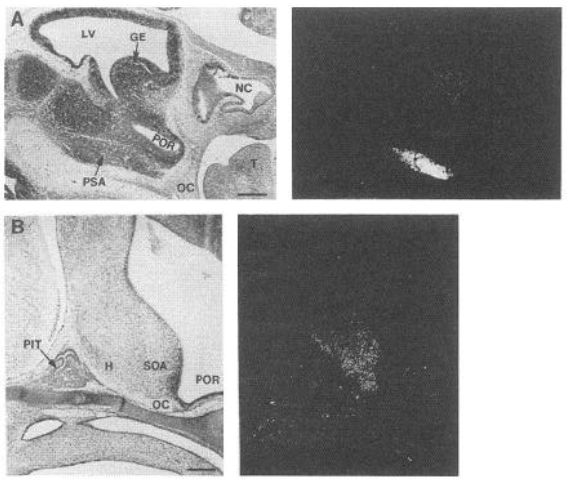

Fig. 8. Expression of SF-1 in the Developing Prosencephalon

Serial sagittal sections from mouse embryos at different developmental stages were prepared and analyzed by *in situ* hybridization, as described in *Materials and Methods*. A, E11.5; brightfield and darkfield analysis with the SF-1 probe. B, E14.5; brightfield and darkfield analyses with the SF-1 probe. *Scale bars* = 120 μm. LV, Lateral ventricle; GE, ganglionic eminence; NC, nasal cavity; POR, preoptic recess; PSA, Postoptic area; OC, optic chiasm; T, tongue; PIT, pituitary; SOA, supraoptic area; H, hypothalamus.

it at two of the three levels of the hypothalamic-pituitary-steroidogenic tissue axis. Recent studies suggest that a number of discrete gene products interact to produce segmental development of the embryonic forebrain (21, 22). Many of these genes belong to the homeobox class of transcriptional regulators; it is intriguing that the mouse homolog of the *FTZ-F1* gene, which is believed to be important for segmentation in *Drosophila* (5), is expressed within one of the few mammalian structures in which segmentation has been implicated. Further studies will be required to determine the spatial and temporal relationship of SF-1 expression with those of other genes implicated in forebrain development. Ultimately, these studies may define a hierarchy of genes that interact to allow the development of complex neuronal structures.

MATERIALS AND METHODS

Materials

Radionuclides were purchased from New England Nuclear-DuPont (Boston, MA). Reagents for *in situ* hybridization were purchased from Novagen (Madison, WI). Reagents for PCR amplification of genomic DNA were purchased from Perkin-Elmer (Norwalk, CT). Restriction and modification enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN).

Animals

All animals were handled in accord with the Guidelines for the Care and Use of Experimental Animals. Timed pregnant Swiss-Webster mice were obtained from Harlan (Indianapolis, IN). Noon of the day on which the copulatory plug was detected was designated day 0.5 of gestation (E0.5). After the mothers were killed by cervical dislocation, the embryos were dissected, fixed in 4% buffered paraformaldehyde at 4 C, and embedded in paraplast. Serial sections of 6 μm were prepared with a microtome. In all cases, the ages of the embryos were confirmed according to the external criteria described by Kaufman (23). The identification of structures in the region of the developing diencephalon was guided by illustrations in the report of Schambra *et al.* (24).

In Situ Hybridization

Serial sagittal sections (6 μm) were deparaffinized and hybridized overnight at 50–55 C using an *in situ* hybridization kit according to the recommended protocol. After washes at high stringency, the slides were dipped in Kodak NTB-2 emulsion diluted 1:1. Exposures were carried out for 3–4 weeks for analyses of E11–E16 sections and 6 weeks for E9–E10 sections. After exposure, slides were developed in Kodak D-19 (Eastman Kodak, Rochester, NY), fixed, and counterstained with methyl green. All experiments included a section of adult mouse adrenal gland to ensure successful detection of the SF-1 and SCC transcripts. No signals above background were observed with any of the sense probes.

Sertoli Cell Preparation

Primary enriched Sertoli cells were isolated from P15 Sprague-Dawley rats, as previously described with minor modification (25). Briefly, rat testes were decapsulated, dispersed in medium 199 supplemented with 20 mM HEPES (pH 7.4) and BSA (0.1% final concentration), and digested twice with collagenase-D (Boehringer Mannheim, Indianapolis, IN) at 0.25 mg/ml for 30 min at 32 C in a shaking water bath. Between digestions, the seminiferous tubules were thoroughly rinsed with Hanks' Balanced Salt Solution and allowed to settle, followed by digestion with hyaluronidase at 0.5 mg/ml (Sigma Chemical Co., St. Louis, MO) for 20 min at 32 C. The pellet was rinsed four times with Hanks' Balanced Salt Solution, and tubules were plated in tissue culture flasks in serum-free F-12 medium supplemented with antibiotics. Medium was changed daily to remove immature germ cells. Sertoli cells were harvested after 4 days, and mRNA was prepared and analyzed by Northern blotting analysis as previously described (26). After transfer to nitrocellulose, the mRNA was probed with a radiolabeled 280-basepair (bp) fragment from the rat SF-1 cDNA (20). Blots were washed at high stringency (0.2 × SSC at 55 C) and analyzed by autoradiography. Alternatively, Sertoli cells were fixed by incubation in 4% buffered paraformaldehyde and used for *in situ* hybridization analysis.

Probes

^{35}S-Labeled cRNA probes for *in situ* hybridization analyses were prepared using T3 and T7 polymerases according to the protocol supplied with a kit purchased from Novagen. Both sense and antisense probes were routinely used in all experiments. The probes used in these experiments include: SF-1, a 200-bp *Acc*I-*Eco*RI fragment derived from the 3′-untranslated region of the SF-1 cDNA that does not hybridize with ELP; and SCC, a 500-bp *Eco*RI-*Bam*HI fragment that includes the 5′-untranslated region and 5′-coding sequences of the mouse SCC cDNA. Additional hybridizations with identical results were performed with a second nonoverlapping SF-1 probe comprising a 270-bp *Sst*I-*Acc*I fragment from the 3′-untranslated region.

Determination of Embryonic Sex by PCR Analyses with *Sry*-Specific Primers

PCR assays were used to determine the sex of the embryos harvested during the indifferent gonad stage. Serial sections

from embryos that did not include the regions of interest were removed from the slides and transferred to microfuge tubes. Genomic DNA was extracted as previously described (27) and subjected to PCR reaction for 70 cycles with oligonucleotide primers derived from the *Sry* sequence. These primers were: *Sry* 5′, 5′-AAGCGCCCCATGAATGCATT-3′; and Sry 3′, 5′-CGATGAGGCTGATATTTATA-3′. PCR products and *Hae*III-digested PhiX174 size markers were analyzed by agarose gel electrophoresis in a 3% NuSieve agarose gel (FMC Bioproducts, Portland, ME). The size of the expected PCR product is 218 bp.

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