# Steroidogenic Factor 1 and Dax-1 Colocalize in Multiple Cell Lineages: Potential Links in Endocrine Development

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**Mutations of the orphan nuclear receptors, steroidogenic factor 1 (SF-1) and DAX-1, cause complex endocrine phenotypes that include impaired adrenal development and hypogonadotrophic hypogonadism. These similar phenotypes suggest that SF-1 and DAX-1 act in the same pathway(s) of endocrine development. To explore this model, we now compare directly their sites of expression. In mouse embryos, SF-1 expression in the urogenital ridge and brain either preceded or coincided with Dax-1 expression, with coordinate expression thereafter in the adrenal cortex, testis, ovary, hypothalamus, and anterior pituitary. The striking colocalization of SF-1 and Dax-1 supports the model that they are intimately linked in a common pathway of endocrine development. The slightly earlier onset of SF-1 expression and its ability to bind specifically to a conserved sequence in the *Dax-1* 5′-flanking region suggested that SF-1 may activate Dax-1 expression. However, promoter activity of Dax-1 5′-flanking sequences did not require this potential SF-1-responsive element, and Dax-1 expression was unimpaired in knockout mice lacking SF-1, establishing that SF-1 is not required for *Dax-1* gene expression in these settings. Although the precise mechanisms remain to be established and may be multifactorial, our results strongly suggest that these two orphan nuclear receptors interact in a common pathway of endocrine development. (Molecular Endocrinology 10: 1261–1272, 1996)**

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## INTRODUCTION

Two orphan members of the nuclear receptor superfamily have recently been shown to play key roles in endocrine differentiation. Steroidogenic factor 1 (SF-1, also called Ad4BP) was first shown to be an essential regulator of the cytochrome P450 steroid hydroxylases (1–3) and was subsequently linked to the expression of the gene encoding Müllerian-inhibiting substance (4, 5). Analyses of mice disrupted in the *Ftz-F1* gene encoding SF-1 revealed adrenal and gonadal agenesis (6, 7), impaired pituitary expression of gonadotrope-specific genes (8, 9), and absence of the ventromedial hypothalamic nucleus [VMH (9, 10)]. These studies collectively demonstrated multiple roles of SF-1 in endocrine differentiation and function.

DAX-1, the second orphan nuclear receptor required for adrenal development (11), was isolated by positional cloning of the gene causing X-linked adrenal hypoplasia congenita (AHC). Patients with AHC present with adrenocortical insufficiency; a subset later develop hypogonadotrophic hypogonadism (reviewed in Ref. 12). Because the gene responsible for this disorder mapped to the DSS critical region of Xp21, which is associated with dosage-sensitive sex reversal in males (13), it was designated *DAX-1* (dosage-sensitive sex reversal-AHC critical region on the X chromosome). The nucleotide sequence of

DAX-1 showed it to be a novel member of the nuclear receptor superfamily, containing sequences homologous to the conserved ligand-binding domain of nuclear hormone receptors but lacking the typical zinc finger DNA-binding motifs. Analyses of affected patients established that DAX-1 mutations cause both X-linked AHC and hypogonadotropic hypogonadism (14). These findings proved that alterations in a single human gene also lead to a compound endocrine phenotype that includes adrenal hypoplasia and impaired gonadotropin release.

The phenotypic similarities that accompany disruption of the *Ftz-F1* gene encoding SF-1 and natural mutations of *DAX-1* suggest that both genes act in the same developmental pathway, determining adrenal development and modulating reproductive function at hypothalamic/pituitary levels. In this paper, we compare the sites of expression of SF-1 and Dax-1 in mouse embryos and adult mice. We also use gel mobility shift and cell transfection assays to examine the possibility that SF-1 regulates Dax-1 expression. Finally, we analyze the effect of targeted disruption of the *Ftz-F1* gene encoding SF-1 on Dax-1 gene expression. Our results strongly suggest that these two orphan nuclear receptors act in a common developmental pathway to effect key events in endocrine differentiation.

## RESULTS

### Dax-1 Expression Colocalizes with That of SF-1

Based on the similar phenotypes associated with mutations in SF-1 and DAX-1, two alternative models seemed most likely: either SF-1 and Dax-1 act sequentially within a hierarchical pathway or the two proteins interact directly to regulate target genes that are critical for endocrine development. The phenotype of *Ftz-F1*-disrupted mice, which includes complete absence of the adrenal glands and gonads, is more severe than that of patients with DAX-1 mutations, suggesting that SF-1 should act upstream of DAX-1 if the sequential model is correct.

Both models predict that SF-1 and Dax-1 expression should colocalize in the relevant endocrine cell lineages during embryonic development and in adult tissues. We therefore used *in situ* hybridization to determine the sites of Dax-1 expression in the adult mouse, comparing these sites with those previously defined for SF-1 (2, 8, 10). As shown in Figs. 1 and 2, Dax-1 transcripts are expressed in a variety of tissues, including the adrenal cortex, testicular Leydig cells, ovarian theca and granulosa cells, the anterior pituitary, and the VMH. Although there was some background, the signals with the sense probe

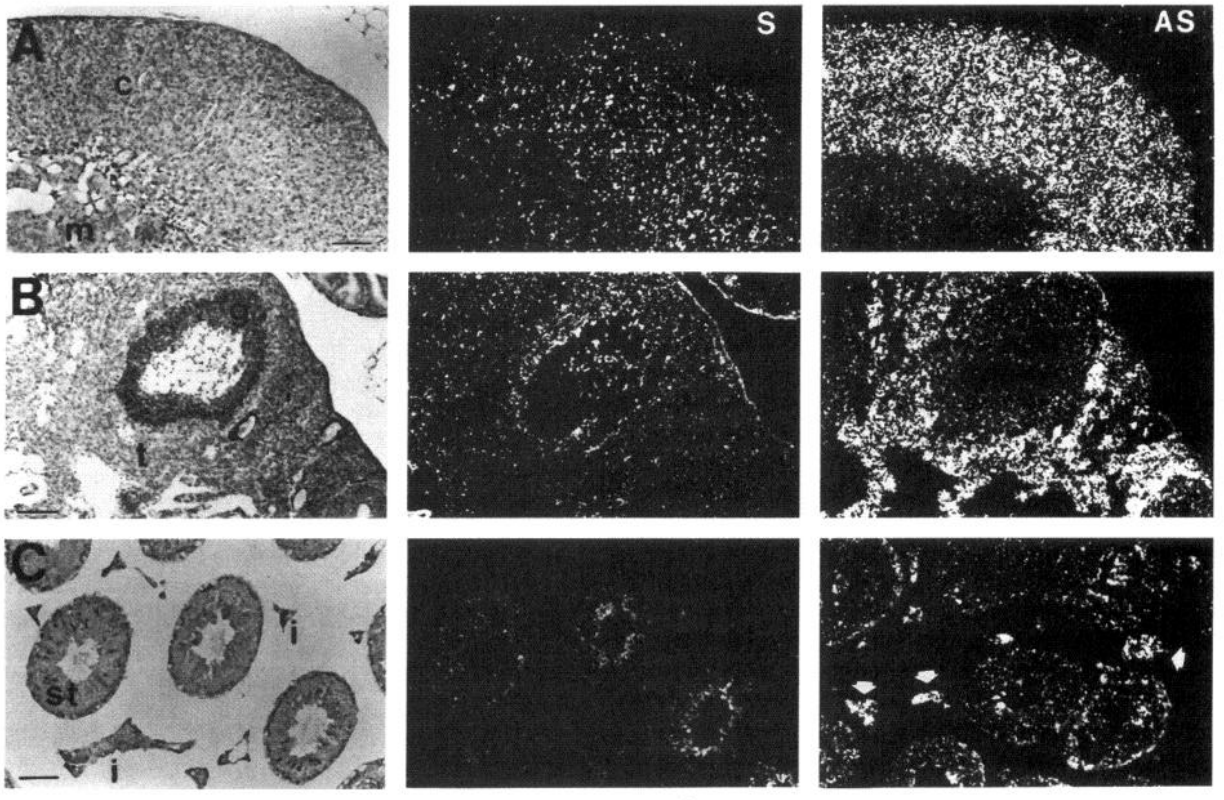


**Fig. 1.** Dax-1 in the Adult Mouse Is Expressed by Multiple Endocrine Cell Lineages

Sites of Dax-1 expression were analyzed by *in situ* hybridization with the Dax-1-specific cRNA probe as described in *Materials and Methods*. Brightfield (*left*) and darkfield sense (*middle*) and darkfield antisense (*right*) views are shown. A, Adrenal gland. B, Ovary. C, Testis. c, Cortex; m, medulla; i, interstitial region; st, seminiferous tubule; t, theca cells. The *arrowheads* in panel C point to the predominant areas of Dax-1 expression in the interstitial region. *Scale bars*, 100 $\mu$m in panels A and B; 50 $\mu$m in panel C.

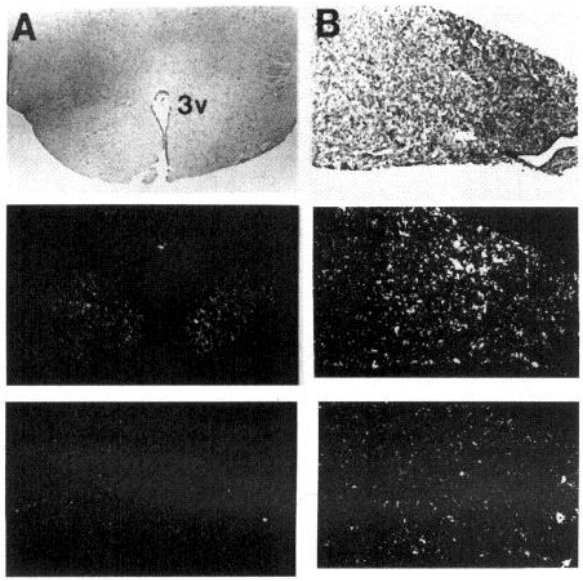


**Fig. 2.** Dax-1 Is Expressed by the Anterior Pituitary and VMH

Sites of Dax-1 expression were analyzed by *in situ* hybridization with the Dax-1-specific cRNA probe as described in *Materials and Methods*. Brightfield (*top*), darkfield antisense (*center*) and darkfield sense (*bottom*) views are shown. A, Hypothalamus. B, Pituitary. 3v, Third ventricle.

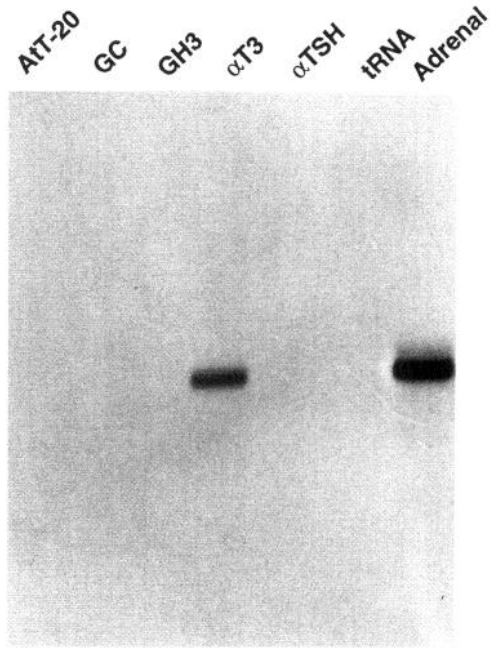


**Fig. 3.** Dax-1 Is Expressed in the Gonadotrope-Derived αT3 Cell Line

RNase protection assays were performed as described in *Materials and Methods*, using the rat Dax-1 probe and total RNA from the indicated cell lines. For reference, a control RNA sample from adrenal glands and a negative control reaction with yeast tRNA were also used in the assay.

did not localize to specific cell types (Figs. 1 and 2). Similarly, no specific signals were obtained when we analyzed a variety of other tissues, including liver, kidney, intestine, lung, and heart (data not shown). In the adult mouse, therefore, there is a striking correspondence between sites of expression of SF-1 and Dax-1.

The anterior pituitary gland contains five discrete cell types, of which only gonadotropes express SF-1 (8, 9). To determine whether Dax-1 is also expressed in gonadotropes, we performed ribonuclease (RNase) protection assays with a probe specific for Dax-1 and RNA prepared from cultured cell lines derived from the different pituitary cell lineages. As expected (Fig. 3), a control sample of mouse adrenal RNA showed a specifically protected band. A similar band was seen with RNA from mouse αT3 cells, which were derived by transgenic immortalization of gonadotropes and express SF-1, strongly suggesting that gonadotropes express Dax-1. In contrast, cell lines derived from other pituitary lineages, including AtT-20 (derived from corticotropes), GC and GH3 (derived from somatomammotrope tumors), and αTSH cells (derived from thyrotropes), did not express Dax-1, strongly suggesting that Dax-1 in the anterior pituitary is selectively expressed by gonadotropes.

### SF-1 and Dax-1 Transcripts Colocalize during Mouse Embryonic Development

Based on the known roles of SF-1 and DAX-1 in adrenal function, we compared their expression during adrenal development. We previously showed that the mouse adrenal primordium is discernible by embryonic day 11 (E11) as a discrete collection of cells that already express SF-1 (15), and that Dax-1 was detected within the adrenal primordium by E12.5 (16). The patterns of expression of Dax-1 and SF-1 are identical at these early stages of adrenal development (Fig. 4). Later, as the sympathoadrenal cells migrate into the adrenal primordium to form the medulla, Dax-1 expression, like that of SF-1, is restricted to the steroidogenic cortical region (16).

Previous analyses of SF-1 expression in the urogenital ridge, which contributes to gonadal, adrenal, and renal development, showed that SF-1 transcripts were detected from approximately E9.0 (15). We recently showed that Dax-1 also is expressed in the genital ridges of both male and female mouse embryos, with expression first detected at E10.5 to E11 (16). Although these results potentially are limited by the sensitivity of the *in situ* procedure, we did not detect Dax-1 transcripts before ~E10.5; thus, the onset of Dax-1 expression in the urogenital ridge may be delayed relative to that of SF-1. The patterns of expression of Dax-1 and SF-1 are indistinguishable in the developing gonads at E10.5 and E11.5, as shown in Fig. 5. Moreover, as the bipotential gonad differentiates into a histologically recognizable testis at approximately E12.5, Dax-1 is expressed diffusely throughout the testis (Fig. 6). This diffuse pattern presumably reflects expression in both compartments of the testis:

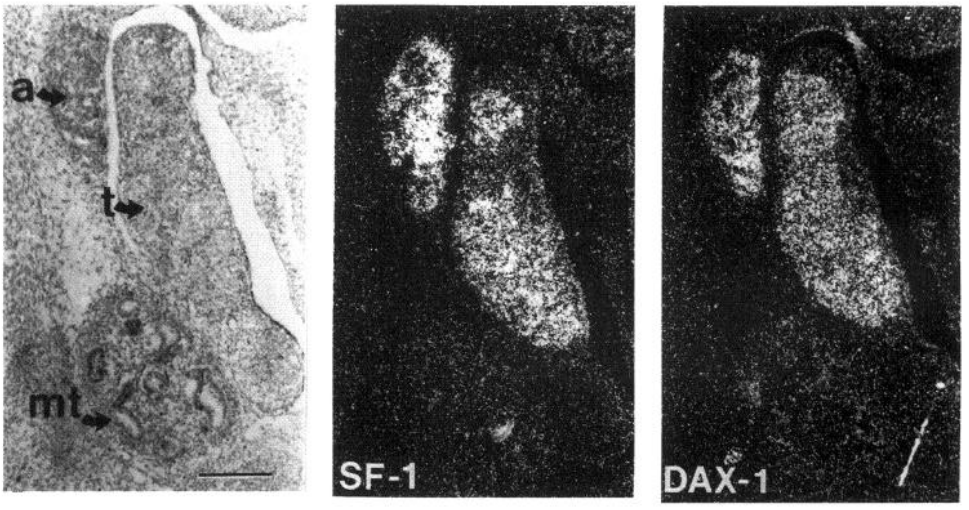


**Fig. 4.** Dax-1 and SF-1 Colocalize at E12. 5 in the Adrenal Primordium and Gonad

Serial sections of an E12.5 male mouse embryo were analyzed by *in situ* hybridization with probes specific for Dax-1 and SF-1 as described in *Materials and Methods*. Shown are the brightfield and darkfield views of the sections hybridized with the Dax-1 probe and the darkfield view of the SF-1 section. a, Adrenal primordium; t, testis; mt, metanephros. *Scale bar*, 200 $\mu$m.

the testicular cords, which contain fetal Sertoli cells and primordial germ cells, and the interstitial region, which contains the steroidogenic Leydig cells. As testicular development proceeds through E14.5, both the SF-1 and Dax-1 signals become restricted to the interstitial region, suggesting that fetal Leydig cells are the predominant site of expression at this time (Fig. 6, *middle*). Finally, by E17.5, the levels of SF-1 and Dax-1 transcripts have diminished considerably, making it impossible to identify expressing cells (Fig. 6, *bottom*).

Although both SF-1 and Dax-1 transcripts are readily detected in the ovary at E12.5 (Fig. 7, *top*), the signals for both transcripts decrease considerably by E14.5 (Fig. 7, *middle*), with an apparent localization of positive cells to the region adjacent to the mesonephros. By E17.5 (Fig. 7, *bottom*), only a very faint signal of Dax-1 expression is seen in the ovary.

Finally, we examined the expression of Dax-1 in the developing diencephalon and anterior pituitary, nonsteroidogenic cells previously shown to express SF-1 (15). As shown in Fig. 8, Dax-1 expression is detected by E11.5 in the same region of the developing brain that expresses SF-1. By E14.5, Dax-1 expression has localized to the retrochiasmatic ventral diencephalon, which ultimately contributes to the hypothalamus, and a weaker signal is also present in the region of the developing anterior pituitary, sites of expression that again are indistinguishable from those of SF-1 (15). Finally, by E18.5, Dax-1 and SF-1 transcripts colocalize to the VMH, the site where they are expressed in the newborn and adult mouse brain.

### The 5′-Flanking Region of the Mouse Dax-1 Gene Contains an AGGTCA Nuclear Receptor Half-Site that Binds SF-1

The 5′-flanking region of the *DAX-1* gene contains a sequence matching the consensus DNA-binding motif for SF-1; this sequence bound SF-1 in gel mobility shift assays (17). After the isolation and characterization of the 5′-flanking region of *Dax-1*, we were intrigued to note that the SF-1-binding site in the human *DAX-1* gene is conserved in the mouse *Dax-1* gene [(TCGAGGTCA at −124, which differs by only one base from the consensus sequence for SF-1 (T/CCAAGGTCA)]. Moreover, gel mobility shift assays (Fig. 9) showed that a protein in nuclear extracts from Y1 adrenocortical cells interacts specifically with this sequence to form a single predominant complex; this complex is specifically competed by unlabeled oligonucleotide that includes the SF-1 site but not by a competitor containing a mutated SF-1 site. Finally, this complex is markedly diminished by pretreatment with a polyclonal antiserum specific for SF-1. These studies, along with the previous analyses of the human *DAX-1* gene (17), raise the possibility that SF-1 directly regulates Dax-1 expression.

### The Putative SF-1-Responsive Element Does Not Regulate Dax-1 Expression in Mouse Y1 Adrenocortical or MA-10 Leydig Cells

To address the possible role of SF-1 and the putative SF-1-responsive element in Dax-1 expression, we performed 5′-deletion analyses with constructs containing varying amounts of Dax-1 5′-flanking sequences placed upstream of a human GH (hGH) reporter gene. These studies were performed in two steroidogenic mouse cell lines, MA-10 Leydig cells and Y1 adrenocortical cells. The MA-10 cells endogenously express Dax-1, along with many of the other components required for steroidogenesis such as the cholesterol side-chain cleavage enzyme. The Y1 adrenocortical cells, in contrast, do not express Dax-1 endogenously but nonetheless provide a well characterized, robust system for studying gene regulation of SF-1-respon-

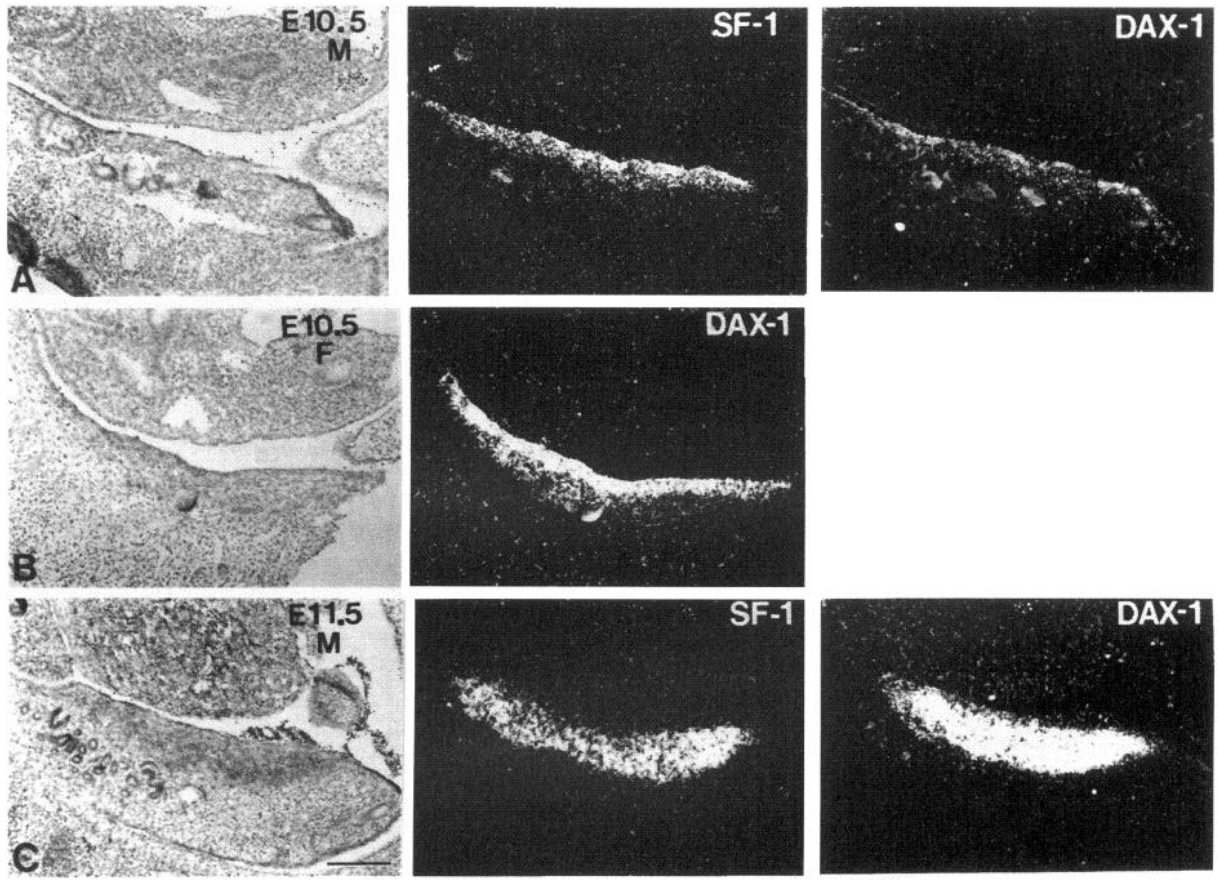


**Fig. 5.** Sites of Expression of Dax-1 and SF-1 during Gonadogenesis in Mouse Embryos

Serial sections of genital ridges from mouse embryos at E10.5 (A and B) or E11.5 (C) were analyzed by *in situ* hybridization with cRNA probes for Dax-1 and SF-1 as described in *Materials and Methods*. Sections in panels A and C were from a male embryo, whereas that in panel B is from a female embryo. Brightfield (*left*) and darkfield (*right*) views are shown. *Scale bar*, 200 $\mu$m.

sive genes. As shown in Fig. 10, deletion of the region containing the potential SF-1-responsive element did not significantly diminish Dax-1 promoter activity in either cell type, suggesting that this sequence is not a major regulator of Dax-1 expression in Leydig or adrenocortical cells. In contrast, deletion of sequences from −72 to −32 markedly impaired Dax-1 promoter activity in both cell lines. Although the specific element responsible for this regulation has not yet been defined, these findings suggest that a critical element for Dax-1 expression is found between −72 and the TATA box at −30. We also performed cotransfection analyses in MA-10 and Y1 cells with an SF-1 expression plasmid (2) and p-563Dax-1/hGH. Although this construct includes the SF-1-binding site at −124, cotransfection with SF-1 had no significant effect on promoter activity (data not shown). Moreover, deletion of the SF-1 binding site at −124 did not decrease promoter activity when 5′-deletion constructs were analyzed in transfected $\alpha$T3 gonadotrope cells (data not shown). While these results do not exclude the possibility that SF-1 regulates Dax-1 expression at key developmental stages *in vivo* or in other cell types, they strongly imply that SF-1 is not important for Dax-1 expression in two different steroidogenic cell lines or in a gonadotrope-derived cell line.

### Dax-1 Expression Persists in the Gonad and Hypothalamus of Ftz-F1-Disrupted Mice

The *Ftz-F1*-disrupted mice, which lack SF-1, provide an excellent way to address *in vivo* the role of SF-1 in regulating Dax-1 expression. We therefore used *in situ* hybridization analyses to examine Dax-1 expression in these knockout mice. Our strategy to disrupt the *Ftz-F1* locus did not interfere with the transcription of SF-1 mRNA (6), allowing us to visualize sites of SF-1 expression by virtue of these transcripts. As shown in Fig. 11A, Dax-1 transcripts are expressed in the *Ftz-F1*-disrupted gonad at E11.5, even though the gonad has already begun to manifest degenerative changes that ultimately lead to its complete disappearance. Similarly, the expression of Dax-1 persists in the developing VMH at E18.5, a time that precedes the disappearance of the VMH in the knockout mice (Fig. 11B). Although they do not exclude the possibility that SF-1 modulates Dax-1 expression to maintain levels

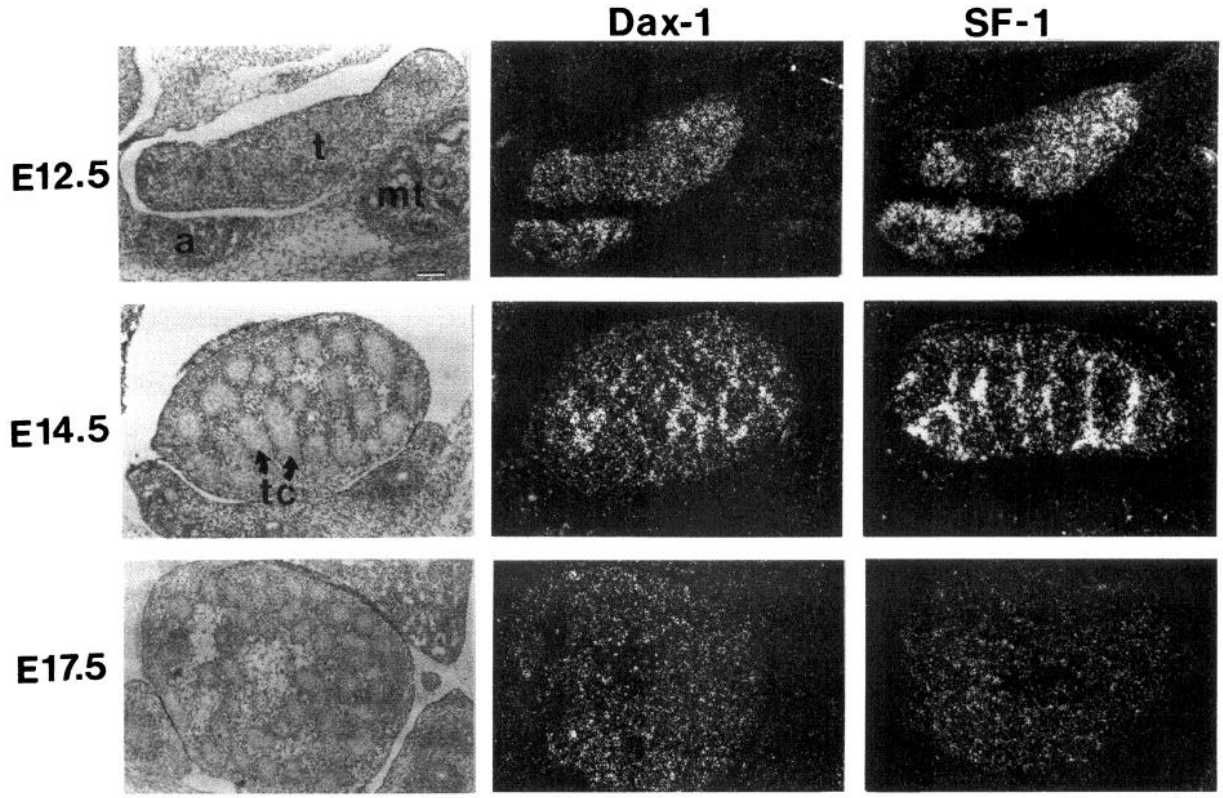


**Fig. 6.** Sites of Expression of Dax-1 and SF-1 at Later Stages of Testicular Development

Serial sections of testes from mouse embryos at the indicated stages of development were analyzed by *in situ* hybridization with cRNA probes for Dax-1 and SF-1 as described in *Materials and Methods*. The E12.5 sections, which are also shown in Fig. 4, are included to present the full time range. Brightfield (*left*) and darkfield (*center and right*) views are shown. t, Testis; a, adrenal primordium; mt, metanephros; tc, testicular cords. *Scale bar*, 100 $\mu$m.

above a critical threshold, these findings demonstrate that SF-1 is not an obligatory positive regulator of Dax-1 expression in the embryonic gonad and hypothalamus.

## DISCUSSION

A critical event in mammalian development is the assumption of a sexually dimorphic phenotype, and considerable attention has been directed at identifying and characterizing the genes that determine sexual determination and differentiation. Although the single critical regulator of male sexual determination is *Sry* (18, 19), it is clear that gonadal development and sexual differentiation also require other genes, and genes encoding the orphan nuclear receptors SF-1 and Dax-1 have recently been implicated in these processes. SF-1 plays multiple roles in mammalian sexual differentiation, acting as an upstream regulator of genes that are required for the production of both essential mediators of male sexual differentiation: androgens and Müllerian-inhibiting substance. SF-1 also is required for testicular and ovarian development, as male and female SF-1 knockout mice completely lack gonads. Dax-1 likewise is expressed at early stages of gonadal development and is implicated in dosage-sensitive sex reversal. These two genes also have been implicated in adrenal development and gonadotropin release, suggesting complex and interrelated roles in endocrine development.

We document here, within multiple cell lineages, a striking colocalization of SF-1 and Dax-1 expression. A recent report using a highly sensitive, but nonquantitative, RT-PCR assay showed that human DAX-1 is expressed by the pituitary and hypothalamus (20). However, these studies provided no information about the precise hypothalamic region(s) or pituitary cell type(s) in which DAX-1 was expressed, and thus fall short of documenting the precise colocalization revealed by our studies. Similarly, although our analyses of the Dax-1 5-flanking region confirmed the previous description of an SF-1-binding site in the DAX-1 promoter region (17), transfection studies in two separate steroidogenic cell lines strongly suggest that this SF-1-binding element is not an important determinant of Dax-1 promoter activity. Finally, Dax-1 transcripts were clearly detectable in the gonads and brain of knockout mice lacking SF-1, showing unequivocally that SF-1 is not an obligatory upstream regulator of *Dax-1* gene expression.

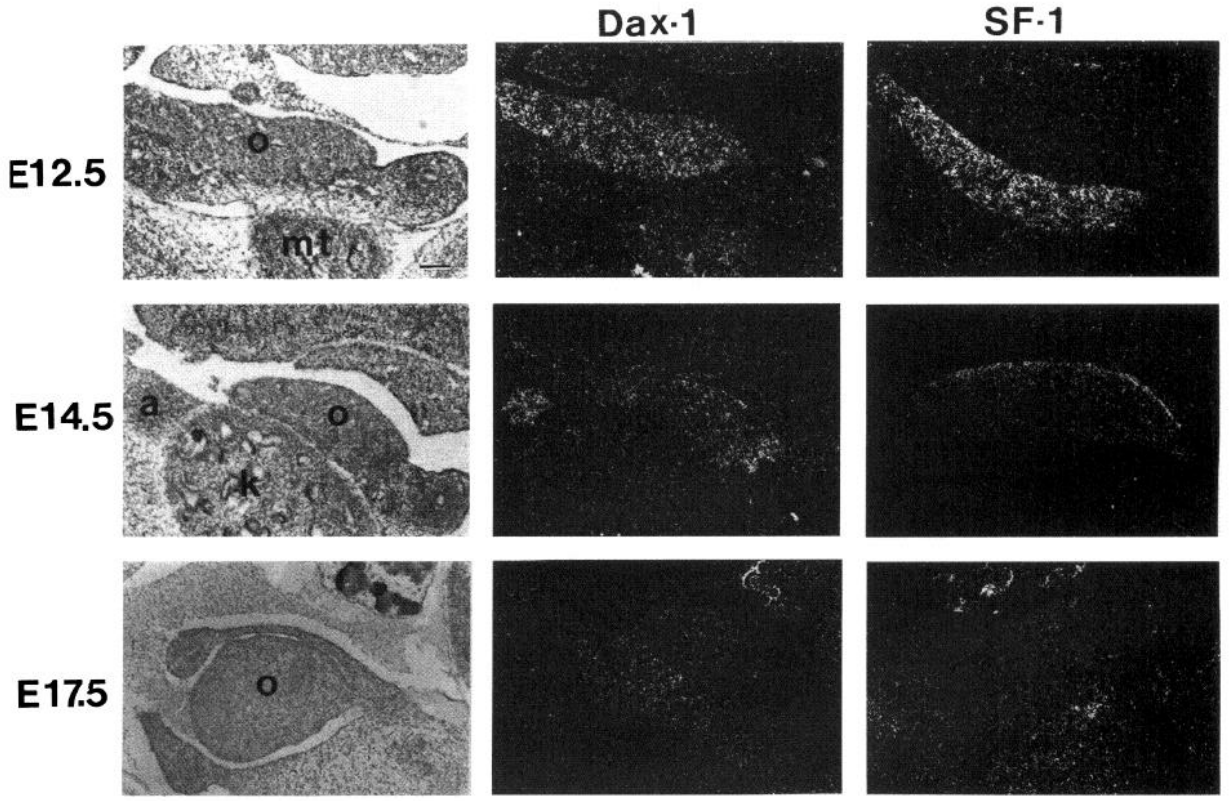


**Fig. 7.** Sites of Expression of Dax-1 and SF-1 at Later Stages of Ovarian Development

Serial sections of ovaries from mouse embryos at the indicated stages of development were analyzed by *in situ* hybridization with cRNA probes for Dax-1 and SF-1 as described in *Materials and Methods*. The E12.5 sections are the same shown in Fig. 4. Brightfield (*left*) and darkfield (*center* and *right*) views are shown. o, Ovary; mt, metanephros; k, kidney; a, adrenal primordium. *Scale bar*, 100 $\mu$m.

Our results also amplify recent analyses of Dax-1 expression during mouse embryological development (16). This study contained no direct analyses of serial sections with the SF-1 and Dax-1 probes, precluding definitive conclusions about colocalization. In addition, Dax-1 transcripts in the anterior pituitary were not shown, and the site of expression in the brain was not localized to the VMH. Thus, the present results provide important new insights about the spatio- and temporal patterns of expression of these two orphan nuclear receptors.

Given that our colocalization data and the similar complex endocrine phenotypes point strongly to a functional interaction of SF-1 and Dax-1, the next step is to define the mechanism(s) by which these genes interact. The available evidence from cell transfection studies and analyses of SF-1-deficient knockout mice argue against a hierarchical pathway in which SF-1 induces Dax-1 expression; conversely, SF-1 expression either precedes or coincides with that of Dax-1, and the SF-1 knockout phenotype is more severe than that seen in patients with DAX-1 mutations, suggesting that Dax-1 does not regulate SF-1 expression. These findings support an alternative model: that SF-1 and Dax-1 interact directly as heterodimers. This model is particularly intriguing because Dax-1 lacks the zinc finger DNA-binding domain conserved among the vast majority of nuclear receptors, while retaining the ligand-binding region that is implicated in dimerization of other superfamily members. Moreover, although SF-1 is generally included in the monomer-binding group of nuclear receptors (20), recent studies have shown that other members of the monomer group, such as NGFI-B, can form heterodimers that are transcriptionally active (22, 23). These findings lend credence to the model that SF-1 and Dax-1 may regulate endocrine development, at least partly, via heterodimerization.

If SF-1 and Dax-1 physically interact, then the functional consequences of their heterodimerization may differ fundamentally in various tissues. Thus, in sites such as adrenocortical cells, gonadotropes, and the VMH, SF-1 and Dax-1 may cooperate to activate the expression of target genes. Consistent with this model, loss-of-function mutations of either gene impair adrenal development and lead to hypogonadotrophic hypogonadism. Moreover, the sequences of both SF-1 and Dax-1 contain regions that correspond to the AF-2 domain (MMLEML in Dax-1 and LLIEML in SF-1). This region, which forms an amphipathic $\alpha$-helical domain, is characteristically found in nuclear receptors that activate transcription (24).

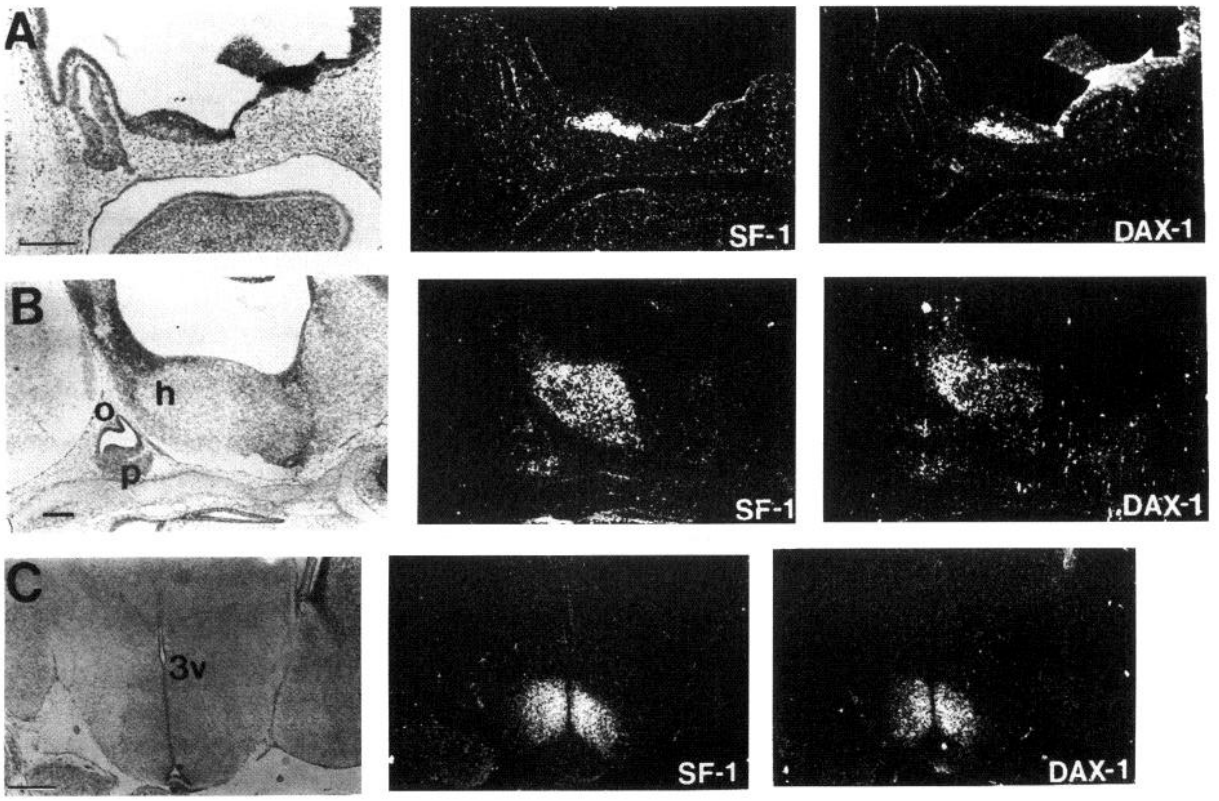


**Fig. 8.** Dax-1 and SF-1 Colocalize in the Developing Brain and Pituitary Gland of Mouse Embryos

Serial sections (sagittal in panels A and B, coronal in panel C) from E11.5 (A), E14.5 (B), and E18.5 (C) mouse embryos were analyzed by *in situ* hybridization with probes specific for SF-1 and Dax-1 as described in *Materials and Methods*. p, Pituitary gland; h, hypothalamus; o, optic chiasm, 3v; third ventricle. *Scale bar*, 200 μm (for A and B); *scale bar*, 500 μm (for panel C).

In contrast, the actions of SF-1 and Dax-1 in gonadal cells during critical periods of sexual differentiation may be antagonistic. SF-1 is required for testes development and male sexual differentiation, whereas its expression in the ovaries diminishes coincident with sexual differentiation; these factors suggest that overexpression of SF-1 might impair ovarian development and female sexual differentiation. In contrast, DAX-1 loss-of-function mutations in males are compatible with normal testicular differentiation, and presumptive gain-of-function mutations in patients with dosage-sensitive sex reversal impair testicular development, findings suggesting that DAX-1 overexpression impairs testicular development and male sexual differentiation.

A final point to consider is the potential modulatory role of ligand(s) in transcriptional activation by SF-1 and Dax-1. To date, no such ligand has been identified; however, both SF-1 and Dax-1 retain conserved regions of the ligand-binding domains of ligand-induced nuclear receptors, and computer modeling predicts that both proteins retain the ligand-binding pocket defined by structural analyses of the retinoic acid receptors (Ref. 25 and P. Chambon, personal communication). It is intriguing to speculate that a ligand modulates the transcriptional activity of SF-1 and Dax-1, perhaps thereby altering the balance between cooperative and antagonistic interactions.

## MATERIALS AND METHODS

### Materials

Radionuclides were purchased from NEN-DuPont (Wilmington, DE). Reagents for *in situ* hybridization were purchased from Novagen (Madison, WI). Reagents for PCR amplification 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 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 0.5 day 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 (26). The *Ftz-F1*-disrupted mice have been reported previously (6, 8, 10). Heterozygous ± animals were mated to produce Ftz-F1 −/− animals. The homozygous

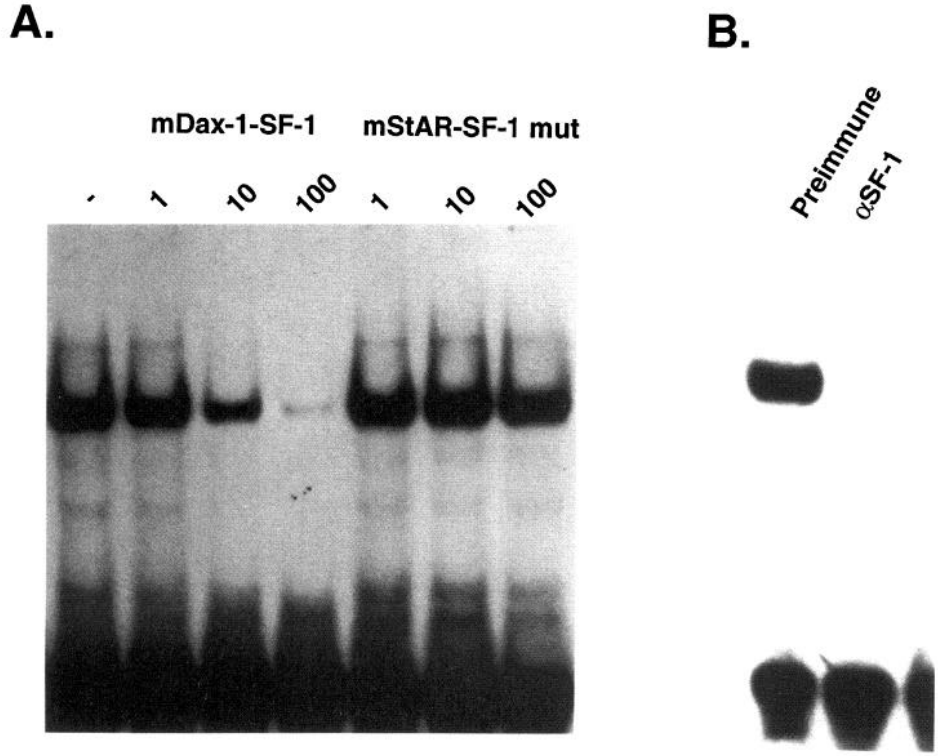


**Fig. 9.** The Mouse *Dax-1* Gene Contains a Potential SF-1-Responsive Element in Its 5′-Flanking Region

A, The putative SF-1-binding site interacts with nuclear extracts from mouse Y1 adrenocortical tumor cells in gel mobility shift assays. Gel mobility shift assays with a labeled probe containing the putative SF-1-responsive element in the Dax-1 5′-flanking region were performed as described in *Materials and Methods*. Unlabeled competitors comprising intact or mutated SF-1-binding sequences were included in the binding reaction at the indicated molar excesses over the labeled Dax-1 probe. B, Anti-SF-1 antiserum specifically abrogates complex formation with the Dax-1 element. Preimmune serum or antiserum raised against the DNA-binding domain of SF-1 was included in the binding reaction as described in *Materials and Methods*.

−/− animals were identified by the absence of adrenals; genotype was confirmed by Southern blotting as described (6). Where indicated, timed pregnancies were set up as detailed above, and embryos were recovered at specific stages of development.

### *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. After exposure, slides were developed in Kodak D-19, fixed, and counterstained with methyl green. All experiments included a section of adult mouse adrenal gland to verify successful detection of the SF-1 and Dax-1 transcripts.

### Probes for *in Situ* Hybridization

$^{35}$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; and Dax-1, a 676-bp RT-PCR-generated fragment of the Dax-1 cDNA that included residues from the putative ligand-binding domain between a *Bam*HI site at position +976 and the TGA stop codon (16).

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

PCR assays were used to determine the sex of 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 and subjected to PCR reaction for 70 cycles with oligonucleotide primers derived from the Sry sequence as previously described (6). These primers were: Sry 5′, 5′-AAGCGCCCCATGAATGCATT-3′, and Sry 3′,5′-CGATGAGGCTGATATTTATA-3′. PCR products were analyzed by agarose gel electrophoresis in a 3% NuSieve agarose gel (FMC, Portland, ME). The size of the expected PCR product is 218 bp.

### RNase Protection Assay

An RNase protection assay was used to measure Dax-1 expression in cell lines derived from different pituitary cell lineages. The probe [prDAX-1 (Pst)] contains rat sequences corresponding to nucleotides 1414 to 1624 of the published human DAX-1 sequence. After linearization with *Eco*RI and probe extension with T7 polymerase, the RNase protection assay was carried out as described (28). The 210-bp protected fragment was resolved by electrophoresis on a 6% denaturing polyacrylamide gel, and radiolabeled species were visualized by autoradiography.

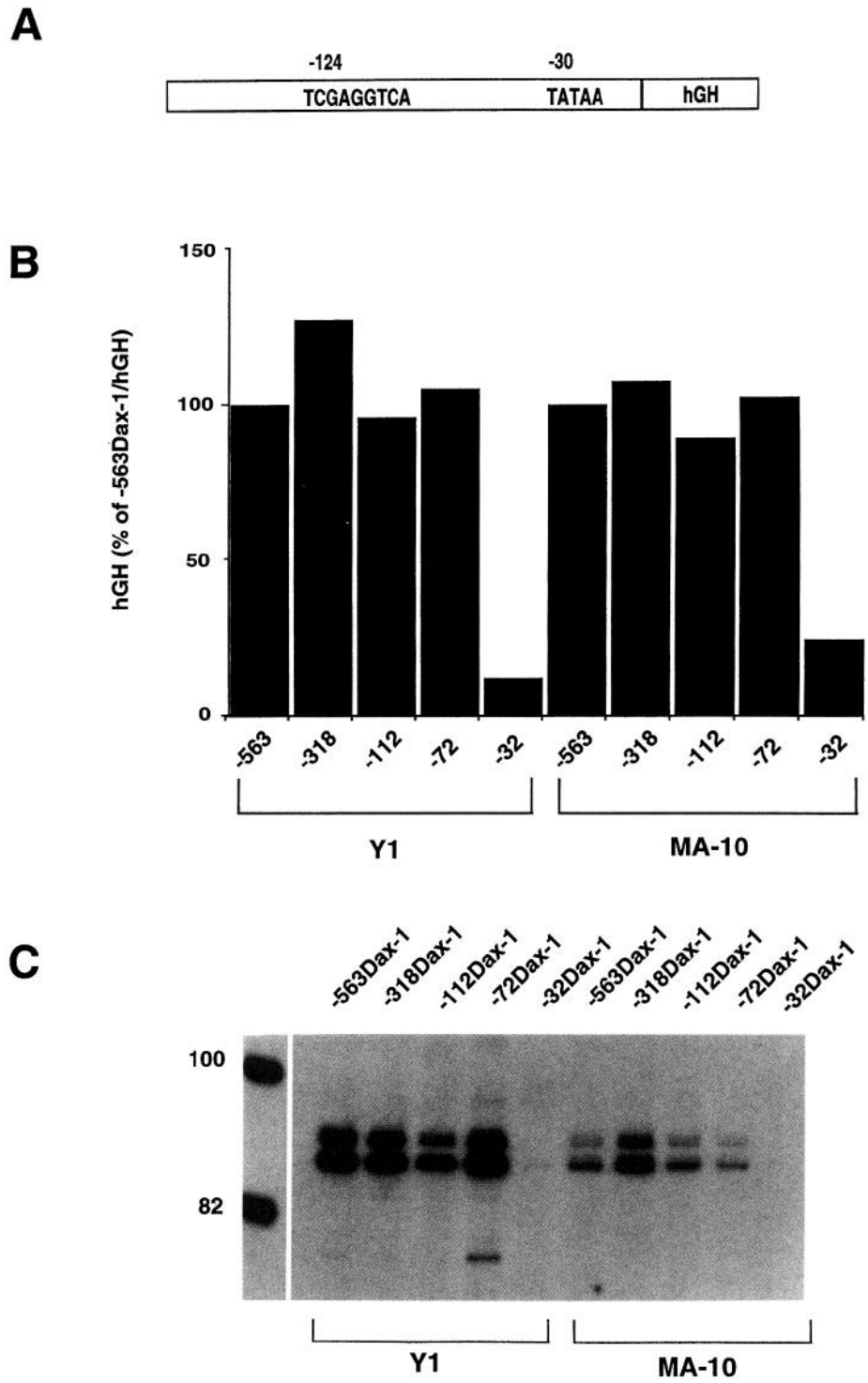


**Fig. 10.** The Potential SF-1-Responsive Element Does Not Regulate Dax-1 Promoter Activity in Transfected Mouse MA-10 Leydig and Y1 Adrenocortical Cells

A, Schematic summary of the Dax-1 5′-flanking region, including the TATA box at −30 and the potential SF-1-responsive element at −124. B, 5′-Deletion analyses of the Dax-1 promoter region-hGH assays. Plasmids containing varying amounts of Dax-1 5′-flanking sequences placed upstream of the human GH (hGH) reporter gene were prepared and transfected into mouse Y1 adrenocortical and MA-10 Leydig cells as described in *Materials and Methods*. Human GH levels for the various constructs are expressed relative to the expression of p-563Dax-1/hGH. All values within each group were within 10% of the mean. C, 5′-Deletion analysis of the Dax-1 promoter region-primer extension analysis. In an independent experiment, the Dax-1 5′-deletion plasmids were transfected into mouse Y1 adrenocortical and MA-10 Leydig cells, and RNA was prepared and analyzed by primer extension analyses as described in *Materials and Methods*. The *numbers on the left* show the positions of mol wt markers of the indicated sizes run on the same gel.

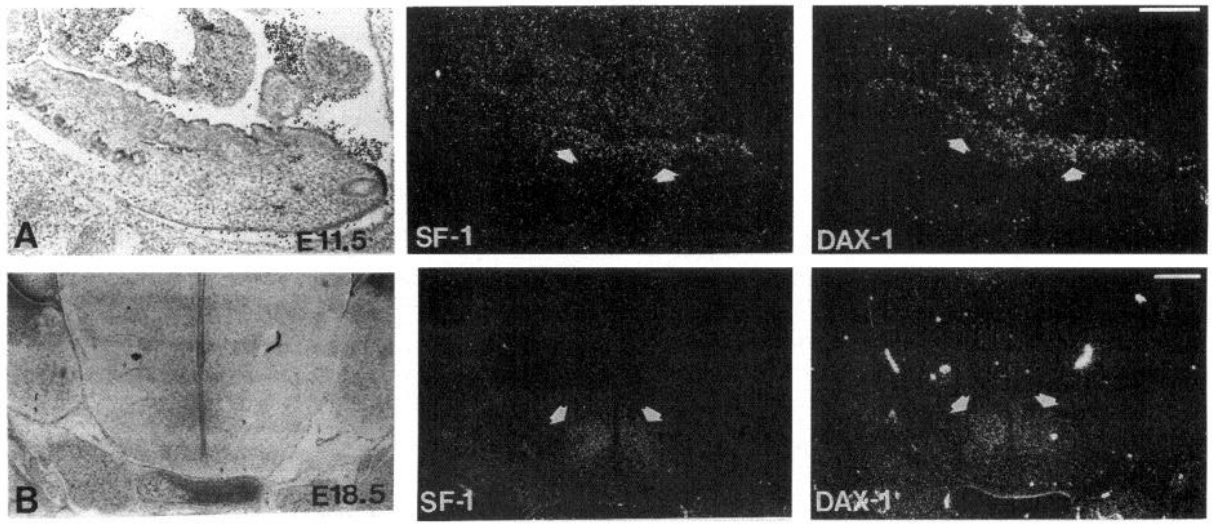


**Fig. 11.** Dax-1 Expression Is Not Abolished in SF-1 Knockout Mice

Serial sections of E11.5 genital ridge (A) or E18.5 hypothalamus (B) from *Ftz-F1*-disrupted mice, which lack SF-1 expression, were analyzed by *in situ* hybridization with cRNA probes for SF-1 and Dax-1. *Arrowheads* indicate sites of expression in the genital ridge (A) or ventromedial hypothalamic nucleus (B). *Scale bar*, 200 $\mu$m in panel A; 500 $\mu$m in panel B.

## Gel Mobility Shift Assays

Nuclear extracts were isolated from the indicated cell lines, and gel mobility shift assays were performed as previously described (1). The probe was prepared by annealing two oligonucleotides to recreate the potential SF-1-responsive site from Dax-1 site −124; *Bam*HI cohesive ends were included to facilitate labeling by Klenow fill-in. The oligonucleotides included: top strand, 5′-GATCCTT**TCGAGGTCA**TGGCCA-3′; bottom strand, 5′-GATCTGGCCA**TGACCTCGA**AAG-3′. Where indicated, unlabeled competitor oligonucleotides at the indicated molar excesses were added to the binding reaction before probe addition. These unlabeled competitors included the potential SF-1 site from the *Dax-1* gene and a mutated form of the SF-1-responsive element that regulates expression of mouse steroidogenic acute regulatory protein (K. Caron and B. Clark, unpublished observation). Experiments using a polyclonal antiserum against the DNA-binding domain of SF-1 were performed as described (2).

## Transfection Experiments

Mouse Y1 adrenocortical and MA-10 Leydig cells were cultured and transfected by the calcium phosphate coprecipitation method as previously described (2). PCR was used to create plasmids (p-563Dax-1/hGH, p-318Dax-1/hGH, p-112Dax-1/hGH, p-72Dax-1/hGH, and p-32Dax-1/hGH) that extended from the indicated 5′-base to an artificial *Bam*HI site just upstream of the Dax-1 initiator methionine. The entire promoter regions of the resulting constructs were sequenced to ensure that spurious mutations were not introduced. Reporter gene expression was determined by RIA for hGH using a kit purchased from Nichols Diagnostics (San Juan Capistrano, CA) or by primer extension with a kinase-labeled oligonucleotide complementary to hGH-coding sequences. The transfection experiment with the 5′-deletion plasmids was performed four times with two different preparations of plasmid DNA for each construct.

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