The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis

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Steroidogenic factor 1 (SF-1), an orphan nuclear receptor, regulates the enzymes that produce sex steroids, and disruption of the *Ftz-F1* gene encoding SF-1 precludes adrenal and gonadal development. We now study the role of SF-1 at other levels of the hypothalamic/pituitary/gonadal axis. In *Ftz-F1*-disrupted mice, immunohistochemical analyses with antibodies against pituitary trophic hormones showed a selective loss of gonadotrope-specific markers, supporting the role of SF-1 in gonadotrope function. In situ hybridization analyses confirmed these results; pituitaries from *Ftz-F1*-disrupted mice lacked transcripts for three gonadotrope-specific markers (LHβ, FSHβ, and the receptor for gonadotropin-releasing hormone), whereas they exhibited decreased but detectable expression of the α-subunit of glycoprotein hormones. SF-1 transcripts in the developing mouse pituitary, which first became detectable at embryonic day 13.5–14.5, preceded the appearance of FSHβ and LHβ transcripts. In adult rat pituitary cells, SF-1 transcripts colocalized with immunoreactivity for the gonadotrope-specific LH. Finally, SF-1 interacted with a previously defined promoter element in the glycoprotein hormone α-subunit gene, providing a possible mechanism for the impaired gonadotropin expression in *Ftz-F1*-disrupted mice. These studies establish novel roles of this orphan nuclear receptor in reproductive function.

[*Key Words*: Nuclear receptor SF-1; reproductive function; gonadal development]

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Gonadal steroids play essential roles in reproduction. Their biosynthesis in the ovary and testis is regulated by complex interactions within the hypothalamic/pituitary/gonadal axis. The predominant regulators of steroidogenesis in gonadal cells are the pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are secreted by gonadotropes in the anterior pituitary. Both mature hormones are heterodimeric glycoproteins containing a common polypeptide, the α-glycoprotein hormone subunit (αGSU), and unique FSHβ or LHβ polypeptide chains (Pierce and Parsons 1981). Gonadotropin release is regulated in turn by gonadotropin-releasing hormone (GnRH), which is produced by neurons in the medial basal hypothalamus and secreted into the hypophyseal plexus in response to various stimuli (Schwanzel-Fukuda et al. 1992). A complex interplay of gonadal steroids, peptides (e.g., activin and inhibin), and binding proteins (e.g., follistatin) mediates feedback regulation at both the hypothalamus and pituitary, altering the overall levels and pattern of gonadotropin release (Gharib et al. 1990).

Development of steroidogenic organs has been shown recently to require an orphan nuclear receptor, designated steroidogenic factor 1 (SF-1), or adrenal 4-binding protein (Lala et al. 1992; Honda et al. 1993). In adult mice, SF-1 is predominantly expressed in the adrenal gland, testis, and ovary, where it regulates the coordinate expression of the cytochrome P-450 steroid hydroxylases (Ikeda et al. 1993). In mouse embryos, SF-1 is found in adrenal and gonadal tissues from their earliest stages of organogenesis (Ikeda et al. 1994). The observed expression of SF-1 in the fetal Leydig cells, which produce androgens, is consistent with its role in regulating the steroidogenic enzymes. We also observed SF-1 expression in fetal Sertoli cells, which produce Müllerian inhibiting substance [(MIS) Ikeda et al. 1994]. A possible rationale for the expression of SF-1 by fetal Sertoli cells came when we implicated SF-1 as an important regulator of the *MIS* gene (Shen et al. 1994). It thus appears that SF-1 regulates both hormones that are essential for male sexual differentiation: androgens and MIS. Our analyses

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also revealed high levels of SF-1 transcripts in the embryonic ventral diencephalon (Ikeda et al. 1994), which gives rise to part of the hypothalamus. Taken together, these observations suggested that the role of SF-1 in reproductive function extends well beyond regulating the enzymes that make gonadal steroids.

To assess directly the role of SF-1 in vivo, we prepared mice with homozygous disruption of the *Ftz-F1* gene, which encodes both SF-1 and a related isoform, the embryonal long terminal repeat binding protein (ELP) (Tsukiyama et al. 1992; Ikeda et al. 1993). All *Ftz-F1*-disrupted animals lacked adrenal glands and gonads and had female internal genitalia (Luo et al. 1994). Analysis of staged *Ftz-F1*-disrupted mouse embryos showed that the gonads initially developed normally but then regressed by programmed cell death (Luo et al. 1994). These findings established the essential role of this gene in steroidogenic organ development and sexual differentiation.

The key role of SF-1 in steroidogenic organ development and its expression in the embryonic diencephalon suggested additional studies to define the function of SF-1 at the level of the hypothalamus and pituitary. In this report we show that the pituitaries of *Ftz-F1*-disrupted mice lack detectable LH, FSH, and GnRH receptor—three gonadotrope-specific markers. We show further that SF-1 is expressed both in normal gonadotropes and in an immortalized gonadotrope cell line, where it interacts with a regulatory element in the αGSU promoter. Our results demonstrate that SF-1 acts at multiple levels of the reproductive axis to permit reproductive competence.

Results

The pituitary glands of Ftz-F1-disrupted mice lack gonadotrope-specific markers

Based on our previous developmental studies showing that SF-1 is expressed at high levels in a region of the embryonic diencephalon that gives rise to the endocrine hypothalamus (Ikeda et al. 1994), we evaluated the effects of *Ftz-F1* disruption at other levels of the reproductive axis. Immunohistochemical analyses of pituitary sections with a panel of antibodies against pituitary trophic hormones revealed a selective deficiency of gonadotrope-specific proteins, with no detectable staining with either the anti-LH or anti-FSH antibodies (Fig. 1A,B). In contrast, *Ftz-F1*-disrupted and wild-type animals showed comparable staining with antibodies against five other pituitary proteins (Fig. 1C–G): Thyroid-stimulating hormone (TSH) expressed in thyrotropes; growth hormone, expressed in somatotropes; prolactin, expressed in lactotropes; adrenocorticotropic hormone (ACTH) expressed in corticotropes; and Pit-1, a transcription factor expressed in cells of the somatotrope, lactotrope, and thyrotrope lineages. The patterns of staining matched the known intracellular locations of the various proteins, with a nuclear distribution of Pit-1 protein and a cytoplasmic localization of the hormones.

Radioimmunoassays for growth hormone confirmed the immunohistochemistry results, showing that circulating growth hormone levels did not differ significantly between *Ftz-F1*-disrupted and wild-type mice (data not shown). The absence of gonadotropins in the *Ftz-F1*-disrupted mice suggests a role for SF-1 in normal pituitary gonadotrope development and/or gene expression.

Next, we performed in situ hybridization analyses with cRNA probes specific for several genes that are expressed in the pituitary. As shown in Figure 2A, proopiomelanocortin (POMC) transcripts, which encode the precursor of ACTH, were expressed in the *Ftz-F1*-disrupted mice at levels at least the equal of those seen in wild-type mice. In contrast, analyses with a probe specific for the αGSU revealed lower, but clearly detectable, levels in the *Ftz-F1*-disrupted mice (Fig. 2B). Given that αGSU is normally expressed in two pituitary cell types, the gonadotropes and thyrotropes, this finding is consistent with the absence of αGSU expression in the gonadotropes with persistent expression at normal levels in the thyrotropes. Consistent with the absence of LH and FSH immunoreactivity, and at a time when they were clearly detected in wild-type littermates, LHβ and FSHβ transcripts were absent from pituitary sections taken from the *Ftz-F1*-disrupted animals (Fig. 2C,D).

Because LHβ and FSHβ are coordinately regulated in gonadotropes and presumably derive from a common ancestral gene (Gharib et al. 1990), we also analyzed levels of transcripts for the GnRH receptor, another gonadotrope-specific marker that shares no structural homology with the gonadotropin β subunits. As revealed by both in situ hybridization (Fig. 3A) and RNase protection assays (Fig. 3B), pituitary expression of the GnRH receptor in *Ftz-F1*-disrupted mice was undetectable when expression was readily detected in wild-type littermates. In the RNase protection assays, β-actin expression was comparable in the +/- and -/- RNA samples, documenting the integrity of the pituitary RNA from the -/- mice. These studies thus indicate that the *Ftz-F1*-disrupted mice are deficient in their expression of three separate gonadotrope-related genes, demonstrating a clear link between *Ftz-F1* (presumably via SF-1) and gonadotrope function.

SF-1 expression during pituitary development temporally precedes the onset of FSHβ and LHβ expression

Previous studies have defined the order in which the markers of different pituitary lineages first appear during development. In the mouse, the onset of expression of the αGSU, which first becomes detectable on embryonic day 12.5 (E12.5), precedes that of FSHβ and LHβ, which are not detected until after E16.5 (Japon et al. 1994). Prompted by the finding that the absence of SF-1 markedly impairs gonadotropin expression, we analyzed the developmental profile of SF-1 transcripts. We did not detect expression in the region of the developing pituitary in any embryos at E12.5 or earlier (data not shown). A faint signal of SF-1 expression in the region of the devel-
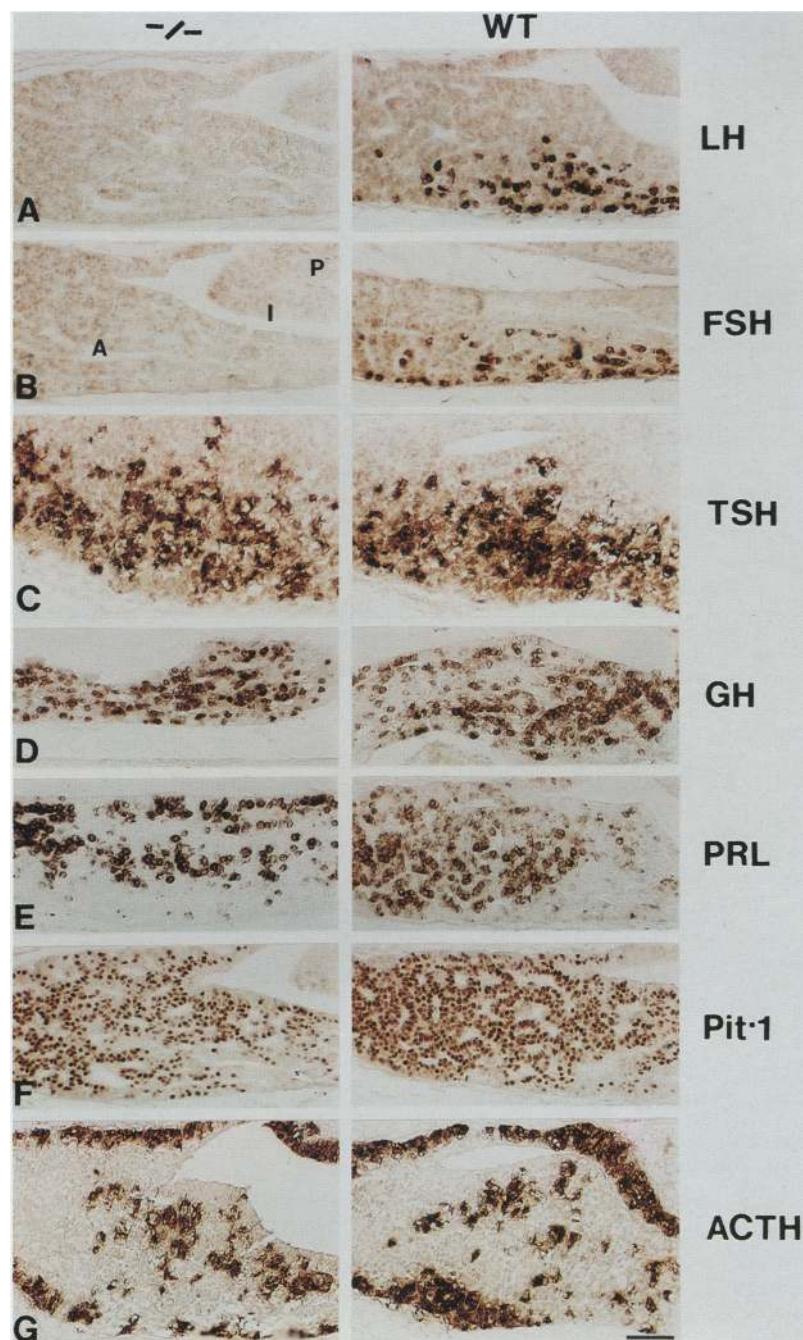
Figure 1. *Ftz-F1*-disrupted animals are selectively deficient in gonadotropin expression. Pituitary glands from *Ftz-F1*-disrupted or wild-type littermates were isolated and analyzed by immunohistochemical staining with antibodies against pituitary trophic hormones and Pit-1 as described in Materials and methods. Representative regions of coronal sections are shown. Bar, 50 μm. (A) Anterior lobe of the pituitary; (I) intermediate lobe; (P) posterior lobe; (LH) luteinizing hormone; (FSH) follicle-stimulating hormone; (TSH) thyroid-stimulating hormone; (GH) growth hormone; (PRL) prolactin; (ACTH) adrenocorticotrophic hormone.

oping anterior pituitary was first detected at E13.5 (Fig. 4). By E14.5, increased levels of SF-1 transcripts were readily detected, with even higher levels at E17.5. Thus, SF-1 is first expressed after the initiation of αGSU expression, indicating that SF-1 is not an obligatory regulator of the αGSU gene at early stages of pituitary development. In contrast, the expression of SF-1 clearly antedates the onset of expression of both LHβ and FSHβ (Japon et al. 1994), consistent with the model that SF-1 regulates their expression directly or indirectly. Similar results were obtained using RNase protection assays

with RNA from rat pituitaries: αGSU expression, which in the rat begins at ~E12.5, preceded the first detectable expression of SF-1 at E14.5, and SF-1 expression, in turn, preceded the onset of LHβ and FSHβ expression at E16.5 (Simmons et al. 1990; H. Ingraham, unpubl.).

SF-1 protein is expressed in the pituitary gland To determine whether SF-1 transcripts detected by in situ hybridization accurately reflected levels of SF-1 protein, we sought to demonstrate SF-1 protein in the pituitary. Because our polyclonal antiserum directed against the
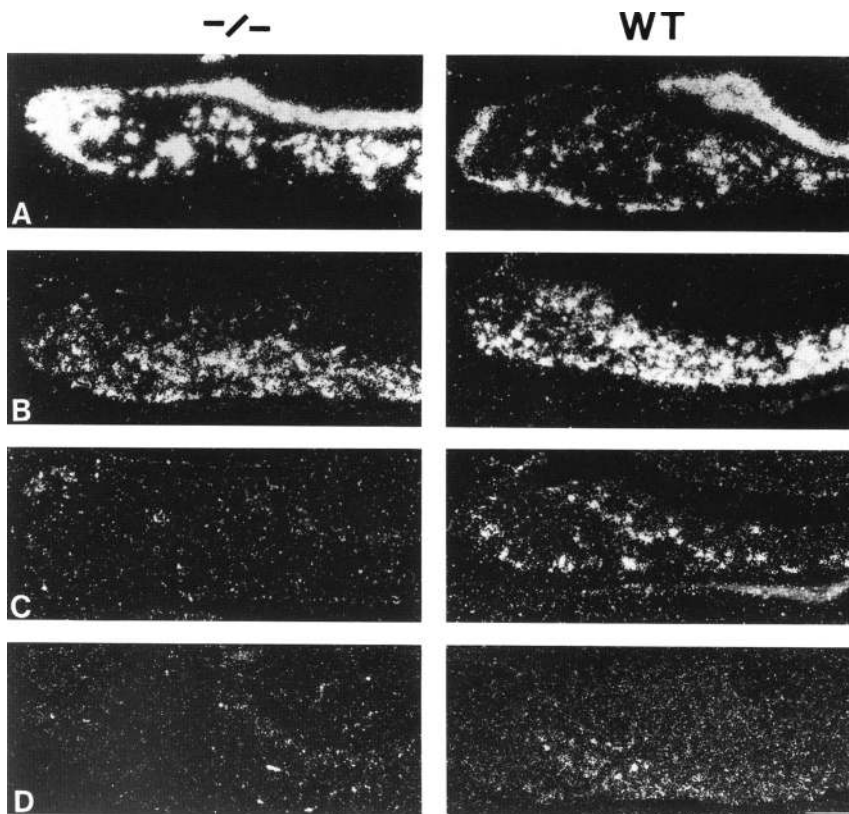
Figure 2. Expressions of mRNAs for the αGSU, LHβ, and FSHβ are diminished in newborn *Ftz-F1*-disrupted animals. Pituitary coronal sections from *Ftz-F1*-disrupted and wild-type littermates were analyzed by in situ hybridization as described in Materials and methods. (*A*) POMC; (*B*) αGSU; (*C*) FSHβ; (*D*) LHβ. Bar, 100 μm.

DNA-binding domain of SF-1 (Ikeda et al. 1993) proved inadequate for immunohistochemical analyses of mouse pituitary sections, we instead used this anti-SF-1 antiserum in immunoblotting analyses of rat pituitary extracts. As shown by the presence of a 54-kD protein (Fig. 5), SF-1 protein is present in rat pituitary extracts but not in extracts derived from rat kidneys. The signal in immunoblotting analyses was corroborated by gel mobility retardation assays, in which both testis and pituitary extracts formed a prominent complex with a probe containing the recently described SF-1 recognition site from the rat MIS gene (Shen et al. 1994). This complex was not formed by kidney extracts and was specifically abolished by preincubation with the anti-SF-1 antiserum (Fig. 5), providing strong evidence that SF-1 protein is expressed in the pituitary. It should be noted that levels of SF-1 protein in the rat pituitary are considerably lower than those seen in adrenal glands or testes, suggesting either that pituitary cells express lower levels of SF-1 or that SF-1 expression is limited to a subset of pituitary cells.

SF-1 is selectively expressed in gonadotropes The selective effect of *Ftz-F1* disruption on gonadotropin expression and the demonstration that SF-1 protein is expressed in the pituitary suggested that SF-1 is expressed in gonadotropes. To test this model, we first examined SF-1 expression in pituitary cell lines derived from the various cell lineages that retain some aspects of their differentiated function. Using RNase protection assays

(Fig. 6), we found SF-1 transcripts only in the gonadotrope-derived cell line αT3-1. This cell line, created by using the 5'-flanking region of *αGSU* to target transgenic expression of the SV40 T antigen, binds GnRH via the GnRH receptor but expresses neither LHβ nor FSHβ-subunits (Windle et al. 1990). In contrast, no SF-1 transcripts were detected in RNA isolated from a hypothalamic cell line expressing GnRH (GT-1) (Mellon et al. 1990) or in other pituitary cell lines representative of thyrotropes (αTSH) (Akerblom et al. 1990), the combined somatotropes/lactotropes (GC), or lactotropes (235-1). In addition, SF-1 was not present in mRNA isolated from the corticotrope cell line, AtT-20 (data not shown).

Immunoblotting analysis with the anti-SF-1 antiserum confirmed that SF-1 protein is present in αT3-1 cells (Fig. 5). Gel mobility retardation assays with a known SF-1-binding site from the steroid 21-hydroxylase gene supported this finding; αT3 extracts produced a prominent complex that migrated identically to the complex seen with Y1 adrenocortical extracts (Fig. 6). This complex migrated identically to that formed by SF-1 produced by in vitro transcription and translation, indicating that SF-1 is very likely the only protein involved in its formation (data not shown). Moreover, in competition experiments with oligonucleotides containing progressive 2-bp mutations of the 21-hydroxylase −140 element, the αT3 protein required the bases CCAAGGCT for efficient binding, matching exactly the binding profile of SF-1 from Y1 adrenocortical cells. Collectively,
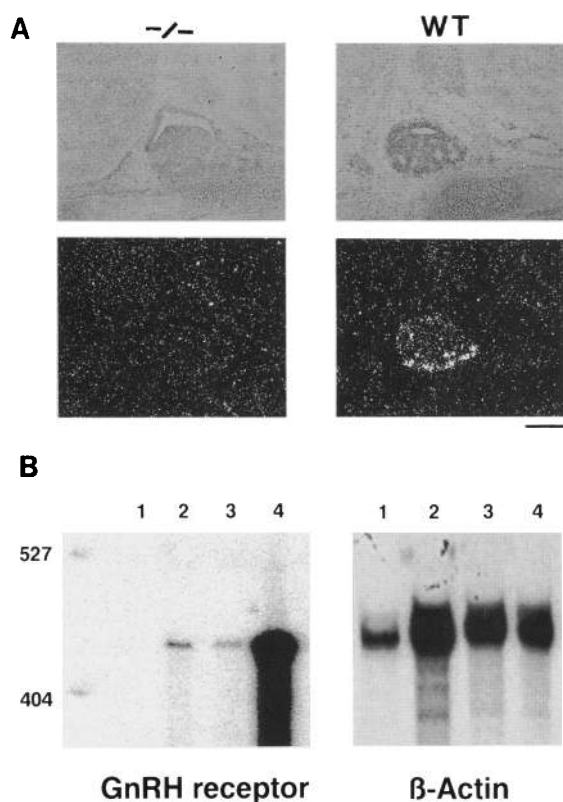
A

B

Figure 3. GnRH receptor transcripts are undetectable in *Ftz-F1*-disrupted animals. (*A*) In situ hybridization analyses. Pituitary sagittal sections from *Ftz-F1*-disrupted and wild-type E17.5 littermates were analyzed by in situ hybridization with the GnRH receptor probe as described in Materials and methods. The scale bar equals 200 μm. (*B*) RNase protection assay of GnRH receptor mRNA levels. Total RNA was prepared and RNase protection assays were performed as described in Materials and methods. The protected fragment of the mouse GnRH receptor probe was 434 nucleotides; the size of the intact probe was 534 nucleotides. (Lane *1*) − / − Pituitary RNA; (lane *2*) + / − pituitary RNA; (lane *3*) + / + pituitary RNA; (lane *4*) αT3 gonadotrope RNA. The positions of size markers are indicated at *left*. (*Right*) An assay performed with a mouse β-actin probe. (Lane *1*) Mouse liver RNA; (lane *2*) αT3 gonadotrope RNA; (lane *3*) + / − pituitary RNA; (lane *4*) − / − pituitary RNA.

these studies show that SF-1, or an immunologically related protein with an identical DNA-binding specificity, is expressed in pituitary gonadotropes but not in other pituitary cell lines.

The αT3 cells are transformed and fail to exhibit important characteristics of gonadotrope function such as LH and FSH expression. We therefore compared the patterns of expression of SF-1 and gonadotrope-specific markers in normal pituitary cells. As shown in Figure 7A, the distribution of SF-1 transcripts in the adult mouse pituitary closely corresponded to the patterns of both FSHβ and LHβ, suggesting that the same cell type (i.e., gonadotropes) expressed all three probes. More definitive support for this model came from combined immunohistochemistry/in situ hybridization analyses per-

formed with primary cultures of rat pituicytes. These experiments (Fig. 7B) clearly showed localization of silver grains representing hybridization to SF-1 mRNA within the darkly labeled cells containing immunoreactive LH. Collectively, these studies show unambiguously that SF-1 is expressed by both SV40-transformed and normal gonadotropes.

SF-1 interacts with the gonadotrope-specific element
Previous studies using αT3 cells identified a conserved promoter element in the 5′-flanking region of the human *αGSU* gene that interacts with a gonadotrope-specific, 54-kD protein (Horn et al. 1992). This promoter element contains an AGGTCA motif matching the known requirements for SF-1 binding (Lala et al. 1992; Morohashi et al. 1992), suggesting that the 54-kD protein may be SF-1. We therefore performed gel mobility retardation assays with radiolabeled gonadotrope-specific element (GSE) and nuclear extracts derived from Y1 adrenocortical, MA-10 Leydig, and αT3 gonadotrope cell lines, as well as several other control cell lines. As shown in Figure 8, indistinguishable complexes were formed by nuclear extracts from Y1 adrenocortical, MA-10 Leydig, and αT3 gonadotrope cells. In addition, the complex formed by nuclear extracts from αT3 cells was inhibited by pretreatment with the anti-SF-1 antiserum or by unlabeled oligonucleotide competitors containing the sequences of two known SF-1 responsive elements (Fig. 8; −140, −210) from the steroid 21-hydroxylase gene (Rice et al. 1991). These findings show that SF-1 is the protein in αT3 cells that binds the GSE and suggest that SF-1 may interact with this element to regulate αGSU expression in gonadotropes.

Discussion

Mammalian reproductive function is regulated by the hypothalamic/pituitary/gonadal axis. We demonstrate here that the nuclear receptor SF-1 is essential for expression of three gonadotrope-specific markers in the pituitary. This finding, combined with the previous observations that SF-1 is essential for gonadal development and function (Ikeda et al. 1994; Luo et al. 1994), implicates SF-1 as a key regulator at multiple levels of reproduction.

The selective loss of gonadotropin expression in *Ftz-F1*-disrupted mice and the presence of SF-1 in αT3 cells and primary gonadotropes suggest that SF-1 in the pituitary is expressed exclusively in gonadotropes. The relatively low level of SF-1 expression observed by in situ analysis is consistent with this, as gonadotropes constitute <10% of cells in the anterior pituitary (Thorner et al. 1992). The dramatic effects of *Ftz-F1* disruption on gonadotrope function most likely reflect either direct actions of SF-1 in gonadotropes or indirect effects that are exerted in the hypothalamus. Within the pituitary, two mechanisms may account for the observed absence of gonadotropin expression in the *Ftz-F1*-deficient mice: either SF-1 is required to establish the gonadotropic lin-
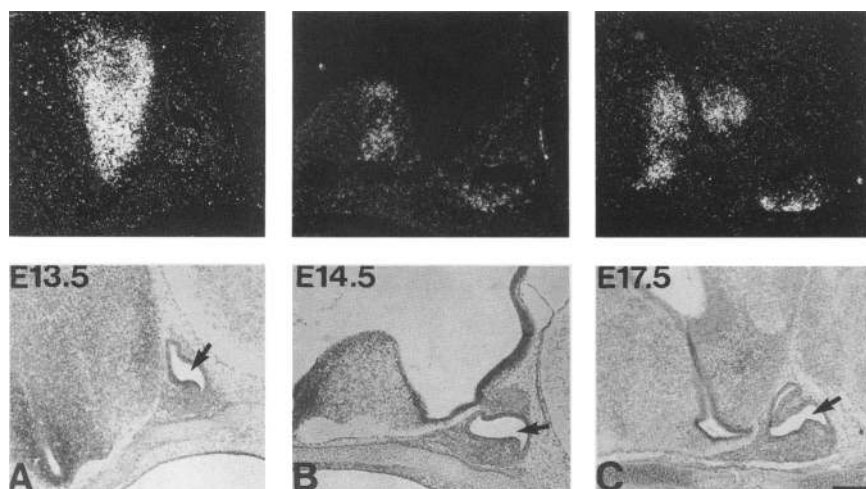
Figure 4. Pituitary expression of SF-1 begins before LHβ and FSHβ are expressed. Sagittal sections of mouse embryos at the indicated stages of development were prepared as described in Materials and methods and hybridized with the SF-1 probe. Bright-field (*bottom*) and dark-field (*top*) views are shown. The arrow indicates the region of the developing pituitary gland. Bar, 200 μm.

eage and/or SF-1 directly regulates the expression of multiple genes required for gonadotropin expression.

At this time, we cannot distinguish between these alternatives. The reduced α*GSU* expression in *Ftz-F1*-disrupted mice and the finding that SF-1 binds a promoter element proposed to regulate α*GSU* expression suggest that SF-1 directly regulates the α*GSU* gene, and by analogy, the LHβ, FSHβ, and GnRH receptor genes. However, the precise role of the GSE site in α*GSU* expression remains undefined. Transfection experiments using αT3 cells suggest that the GSE is important for α*GSU* expression (Horn et al. 1992). Moreover, the GSE lies within the proximal 313-bp region of 5'-flanking sequences shown to direct gonadotrope-specific expression in transgenic targeting experiments (Kendall et al. 1991; Hamernik et al. 1992). However, it is unclear whether the GSE–SF-1 interaction fully accounts for gonadotrope-specific expression in vivo, as mutations of the GSE in transfection studies result in relatively small diminutions in expression of reporter genes (Horn et al. 1992; J. Nilson, unpubl.). Full levels of α*GSU* expression likely

depend on combinatorial regulation, and other promoter elements almost certainly contribute to its expression. One such element recently implicated in pituitary α*GSU* expression interacts with the LH-2 LIM–homeodomain factor (Roberson et al. 1994).

Given that pituitary SF-1 transcripts first appear slightly before the onset of expression of LHβ and FSHβ and that *Ftz-F1*-disrupted mice lack LHβ and FSHβ expression, SF-1 may also directly regulate the LHβ and FSHβ genes. To date, no studies have implicated potential SF-1-binding sites in the regulation of the LHβ and FSHβ promoters. The absence of such sites, if supported in subsequent studies, would argue that SF-1 effects on β-subunit expression are indirect, or alternatively, that SF-1 is necessary for development of the gonadotrope cell lineage.

Is SF-1 directly required for development of the gonadotropes in the pituitary? Precedence exists for dual involvement of a single transcription factor in development of specific pituitary cells types and expression of cell-specific markers. Pit-1 (GHF-1) is directly impli-
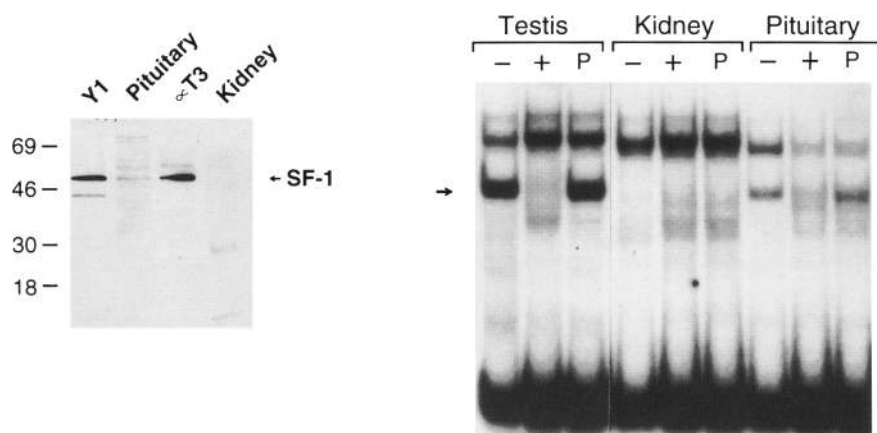

Figure 5. Pituitary glands contain SF-1 protein. Rat pituitaries were isolated and nuclear extracts were prepared as described in Materials and methods. (*Left*) Immunoreactive SF-1 is expressed in the rat pituitary. Immunoblotting analyses of rat pituitary extracts from P20 rats (Pituitary) were performed as described in Materials and methods. The arrow indicates the immunoreactive, 54-kD pituitary protein that migrates identically to authentic SF-1 from Y1 adrenocorticol cell extract (Y1) and in extracts from the gonadotrope cell line, αT3-1 (αT3). (*Right*) Anti-SF-1 abrogates the complex formed with the pituitary extracts. Equivalent amounts of extracts (3 μg of protein) were reacted alone (−), with a specific anti-SF-1 antiserum (+), or with preimmune serum (P) as described in Materials and methods. The specific SF-1–DNA complex is indicated (→).

Mutation #   1 2 3 4 5 6 7

Sequence   CCACAGATTCT CCAAGGCT GATGG

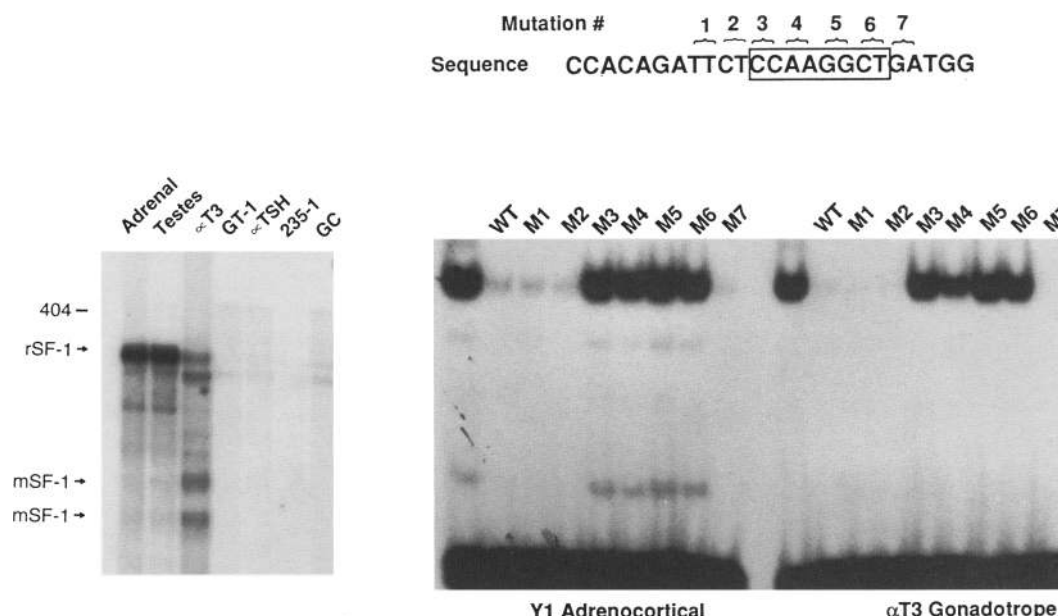Y1 Adrenocortical   αT3 Gonadotrope

Figure 6. αT3 gonadotropes contain SF-1. (*Left*) RNase protection assays were carried out as described in Materials and methods. Samples include control rat adrenal and testes RNA from P0 rats and the mouse αT3-1 cell line (αT3), the mouse GT-1 hypothalamic cell line (GT-1), mouse αTSH thyrotrope cell line (αTSH), the rat 235-1 prolactinoma cell line (235-1), and the rat somatomammotrope cell line (GC). Protection patterns using rat vs. mouse RNA are indicated by arrows. (*Right*) Demonstration that the αT3 protein has an identical binding specificity to SF-1 in Y1 adrenocortical cells. Gel mobility retardation assays were performed with the 21-hydroxylase −140 probe, a known high-affinity SF-1-binding site, and nuclear extracts from Y1 adrenocortical and αT3 gonadotrope cells. Where indicated, either wild-type unlabeled competitor or various mutated competitors containing 2-base alterations of the wild-type sequence were included. The bases altered in the various mutated competitors are shown (*top*); those bases essential for binding are boxed.

cated in the development and function of three pituitary cell types: somatotropes, lactotropes, and thyrotropes (Bodner et al. 1988; Ingraham et al. 1988; for review, see Voss and Rosenfeld 1992; Theill and Karin 1993). Certain natural mutations of Pit-1 lead to pituitary hypoplasia and deficiency of all three cell types (Li et al. 1990), whereas other mutations affect somatotropes and lactotropes without impairing thyrotrope function and, thus, are not associated with marked pituitary hypoplasia (Pfäffle et al. 1992). Loss of SF-1, like Pit-1, has been shown to result in the loss of progenitor cells in adrenals and gonads by programmed cell death (Luo et al. 1994). Further analysis of pituitaries within these *Ftz-F1*-disrupted mice may reveal a similar loss of the gonadotrope lineage. Alternatively, it remains possible that cells derived from the gonadotrope lineage persist in the pituitaries of the knockout mice but are unable to express known markers of gonadotrope function, including gonadotropins. Distinguishing between these possibilities may ultimately require the isolation of additional markers of gonadotrope lineage that are not directly regulated by SF-1. In this regard, the finding that GnRH receptor expression is also impaired in *Ftz-F1*-disrupted mice (Fig. 3) suggests that such markers may be difficult to identify.

The expression of SF-1 in the ventral diencephalon, which ultimately contributes to the endocrine hypothalamus, suggests a direct or indirect role for SF-1 in the GnRH hypothalamic neurons (Ikeda et al. 1994). Thus, it is attractive to propose that SF-1 acts at all three levels of the hypothalamic/pituitary/gonadal axis. We have not yet identified the specific hypothalamic cells expressing SF-1 and do not know whether SF-1 is found in GnRH neurons. To the extent that the GT-1 cell line accurately reflects GnRH neuron function, their lack of SF-1 expression suggests that GnRH neurons do not express SF-1. It remains possible that SF-1 is normally present in GnRH neurons but that GT-1 cells lack the full repertoire of differentiated functions in a manner akin to other immortalized cell lines generated by transgenic targeting of SV40 large T oncoprotein (Windle et al. 1990; Lew et al. 1993; Peschon et al. 1992). If SF-1 does act primarily at the level of the hypothalamus to affect gonadotrope function, then it may be possible to rescue gonadotropin expression in *Ftz-F1*-disrupted mice. Similar GnRH rescue studies have been successful in the hypogonadal mouse, which has gonadotrope defects secondary to a mutation in the *GnRH* gene (Charlton et al. 1983; Mason et al. 1986).

The phenotype of the *Ftz-F1*-disrupted mice includes both adrenal/gonadal hypoplasia and selective deficiency of gonadotropin expression. Intriguingly, a very similar clinical presentation is seen in a subset of human patients with X-linked adrenal hypoplasia, who also have decreased gonadotropins and gonadal dysfunction in combination with adrenal hypoplasia (Kletter et al.
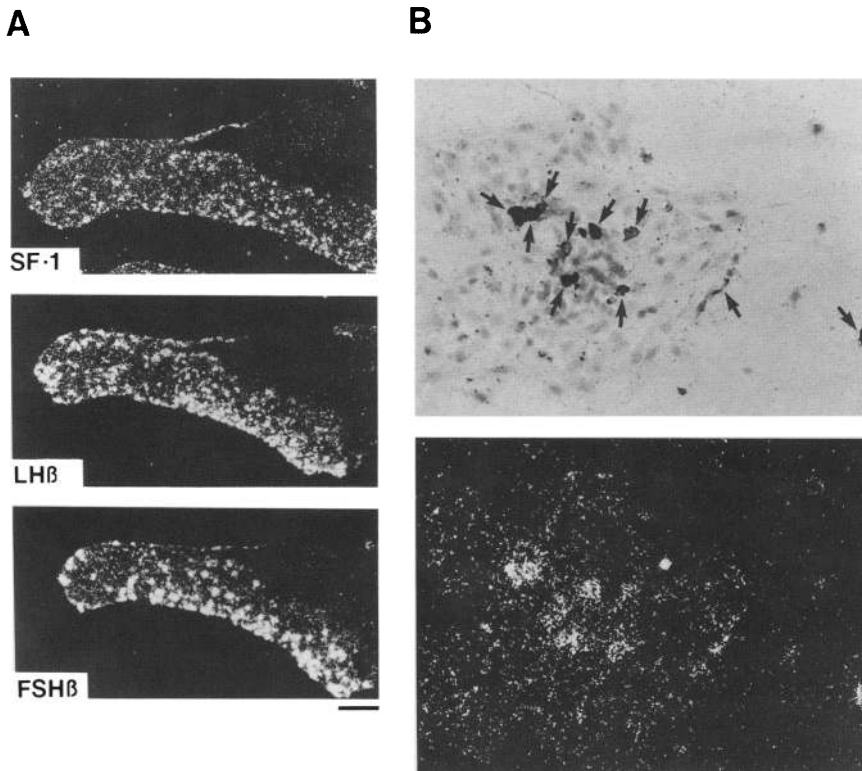
A

B

Figure 7. SF-1 colocalizes with gonadotropins. (*A*) SF-1, LHβ, and FSHβ transcripts are expressed in very similar patterns in the anterior pituitary. Serial sections of the anterior pituitary from an adult female mouse were analyzed by in situ hybridization with the indicated probes as described in Materials and methods. Dark-field photomicrographs with the SF-1, LHβ, and FSHβ probes are shown. (*B*) SF-1 transcripts and LH immunoreactivity colocalize in primary cultures of rat pituicytes. Primary cultures of rat pituitary cells were prepared and analyzed by combined immunohistochemical analyses with an antiserum specific for LH and in situ hybridization with an SF-1 cRNA probe as described in Materials and methods. The arrows indicate cells that contain LH immunoreactivity (dark color in bright-field view), which colocalize with silver grains indicative of SF-1 mRNA (bright areas in dark-field view). Bar, 200 μm (*A*); 50 μm (*B*).

1991). It is unlikely that SF-1 mutations cause this X-linked syndrome because the human homolog of SF-1 maps to chromosome 9 (K. Parker and C. Morton, unpubl.) and *Ftz-F1*-disrupted mice completely lack gonads, whereas human patients have poorly developed but detectable testes. The striking similarities in these complex endocrine phenotypes nonetheless suggest that SF-1 and the gene product underlying X-linked adrenal hypoplasia are linked in a common developmental pathway.

Our analyses of *Ftz-F1*-disrupted mice (this paper; Luo et al. 1994) establish the role of SF-1 in the development and function of multiple endocrine organs that are essential for reproduction. Further identification of genes that act upstream and downstream of SF-1 in the complex events of reproductive organ development and characterization of ligands or coactivators that modulate SF-1 transcriptional effects will undoubtedly provide novel insights into the mechanisms of endocrine differentiation and reproductive function.

Materials and methods

Materials

Restriction and modification enzymes were obtained from New England Nuclear (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Radionuclides were purchased from New England Nuclear/E.I. DuPont (Boston, MA). Antibodies specific for TSH, LH, FSH, ACTH, and GH were provided by Drs. Susan Greenhut and A. F. Parlow through the National Hormone and Pituitary Program. Antibody against human prolactin was purchased from Lipshaw Immunologicals (Pittsburgh, PA). Reagents for in situ hybridization were purchased from Novagen (Madison, WI). Reagents for immunohistochemical analyses were purchased from Vector Laboratories, Inc. (Burlingame, CA) or Sigma Chemical Co. (St. Louis, MO). The αT3 cells were provided by Dr. Pamela Mellon (University of California, San Diego), and the MA-10 cells were provided by Dr. Mario Ascoli (University of Iowa, Iowa City).

Ftz-F1-disrupted animals

Mice that are homozygously disrupted at the *Ftz-F1* locus have been reported recently (Luo et al. 1994). Briefly, a neomycin phosphotransferase selectable marker was inserted within the shared exon encoding the second zinc finger region (Ikeda et al. 1993), thus interfering with function of both the SF-1 and ELP isoforms of *Ftz-F1*. *Ftz-F1* −/− animals were produced by breeding heterozygous +/− animals, which are reproductively intact. The homozygous −/− animals were identified by the absence of adrenals; genotype was confirmed by Southern blotting as described (Luo et al. 1994).

In situ hybridization and immunohistochemistry

Serial 6-μm sections of dissected pituitaries from wild-type and *Ftz-F1*-disrupted newborn mice 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. Exposure times were 1 week for analyses with the rat αGSU (Godine et al. 1982), mouse POMC (Uhler and Herbert 1983), and rat FSHβ (Maurer 1987) probes, 3 weeks for the mouse GnRH receptor probe (Tsutsumi et al. 1992), and 4–6 weeks for analyses with the rat LHβ (Tepper and Roberts 1984) and mouse SF-1 probes (Ikeda et al. 1993). Following exposure, slides were developed in Kodak D-19, fixed, and counterstained
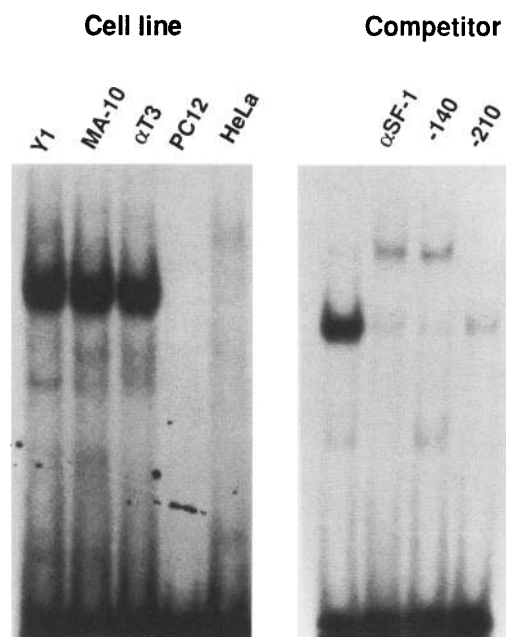
Cell line   **Competitor**

Figure 8. SF-1 is the αT3 protein that interacts with the GSE. (*Left*) A GSE-containing complex of similar mobility is formed by Y1 adrenocortical, MA-10 Leydig, and αT3 gonadotrope nuclear extracts. Nuclear extracts from the indicated tissues were prepared and gel mobility retardation assays with the GSE probe were performed as described in Materials and methods. A prominent complex was seen with Y1, MA-10, and αT3 extracts. (*Right*) The GSE complex is specifically inhibited by antiserum against SF-1 or by known SF-1-binding sequences. Gel mobility retardation assays with the GSE probe and αT3 nuclear extracts were performed. Where indicated, extracts were preincubated with unlabeled competitors containing either the 21-hydroxylase −210 or −140 elements or with antiserum against SF-1 (αSF-1).

with methyl green. No signals above background were observed with any of the sense probes.

For the GnRH receptor studies, in situ hybridization analyses used a digoxygenin-labeled, 416-nucleotide probe corresponding to a 416-bp PstI–PstI fragment of the mouse GnRH receptor. The plasmid was linearized with EcoRI, and probe was synthesized with T7 RNA polymerase in the presence of digoxygenin-11-UTP. After hybridization, the signal was detected by incubating with alkaline phosphatase-coupled anti-digoxygenin antibody (1:1000 dilution), followed by color reaction using nitoblue tetrazolium salt, 5-bromo-4-chloro-3-indol-phosphate toluidinium salt, and levamisol to give blue staining.

In immunohistochemical analyses, paraformaldehyde-fixed, paraffin-embedded 6-μm sections were used. The primary antibodies and the dilutions at which they were used included rabbit anti-human ACTH (1:4000), guinea pig anti-rat LH (1:3000), guinea pig anti-rat FSH (1:1000), rabbit anti-rat TSH (1:4000), monkey anti-rat growth hormone (1:15000), and rabbit anti-Pit-1 (1:1500). To detect the peroxidase reaction, a Vectastain ABC kit was used, except with the anti-growth hormone antibody, where an anti-monkey IgG peroxidase conjugate was purchased from Sigma. Control reactions were performed routinely by substituting normal rabbit serum for the primary antibody.

Colocalization studies combining immunohistochemical

analyses with in situ hybridization were performed with primary cultures of rat pituitary cells. Primary cultures of rat pituitary cells were prepared as described (Simmons et al. 1990). Immunohistochemical analyses with the anti-LH and anti-FSH antibodies and in situ hybridization with the SF-1 antisense probe were performed essentially as described above.

Gel mobility retardation assays

Nuclear extracts were prepared from various cultured cell lines as described (Shapiro et al. 1988). Gel mobility retardation assays with the 21-hydroxylase −140 and −210 probes were performed as described (Rice et al. 1991), and those with the MIS SF-1-responsive element were done as reported previously (Shen et al. 1994). The GSE probe contained bases from −222 to −193 of the mouse αGSU 5'-flanking region, matching the region identified previously as the GSE (Horn et al. 1992), with HindIII ends added to facilitate labeling (top strand, 5'-agctCATAAGC-TGTCCTTGAGGTCACCACTACCT-3'). Where indicated, preimmune or immune anti-SF-1 antiserum was added to the binding reaction for 30 min before addition of probe in the gel mobility retardation assays as described previously (Ikeda et al. 1993). To define the bases required for DNA–protein interaction, we performed gel mobility retardation assays with nuclear extracts from Y1 adrenocortical or αT3 cells and unlabeled oligonucleotide competitors that included either the wild-type 21-hydroxylase −140 element or mutations M1–M7 containing the indicated 2-base alterations of the native sequence (Lala et al. 1992).

Immunoblotting

Nuclear extracts from various tissues or dissected pituitaries were resolved by SDS-PAGE, transferred to a nitrocellulose membrane by electroblotting, preincubated for 1 hr in TBS, 1% Tween-20, and 5% nonfat dry milk, and washed repeatedly in the same buffer containing 1% milk. The filter was incubated overnight at 4°C with rabbit anti-SF-1 polyclonal antibody (1:5000 dilution), washed, and incubated for 1 hr with the secondary antibody (goat anti-rabbit horseradish peroxidase conjugate, 1:3000 dilution). After washing, the blot was developed with a Chemiluminescence kit (New England Nuclear–DuPont) and exposed to Kodak X-Omat film.

RNase protection assays

RNase protection assays with the rat SF-1 probe were performed as described previously (Shen et al. 1994), with minor modifications. The rat SF-1 cRNA probe was generated by linearizing the partial rat SF-1 cDNA (Lynch et al. 1993) with NcoI and transcribing with an RNA transcription kit (Stratagene) and T3 polymerase (Promega). The probe was gel purified and precipitated, and 500,000 cpm of the labeled probe was mixed with 5 μg of RNA samples, denatured at 95°C, and hybridized overnight at 55°C. Samples were digested in a 330-μl volume with 10 μg of RNase A (Sigma) and 0.5 μg of RNase T1 (Boehringer Mannheim) for 60 min at 37°C, treated with 20 μg of proteinase K (Boehringer Mannheim) in the presence of carrier tRNA, and analyzed on a 6% 0.5× TBE denaturing polyacrylamide gel. Total RNA was isolated as described (Moore et al. 1990).

For experiments with the GnRH receptor, a plasmid containing a 592-bp HindIII–HindIII fragment of the mouse GnRH receptor (Tsutsumi et al. 1992) was linearized with StuI and extended with T7 polymerase to generate a 534-nucleotide antisense probe containing 434 nucleotides of GnRH receptor sequence. This probe was used in RNase protection assays with

total pituitary RNA (10 μg) prepared from +/+, +/−, and −/− mice and an RNase protection kit purchased from Ambion (Austin, TX). The integrity of the RNAs was confirmed by RNase protection assays with a probe for mouse β-actin supplied with the kit.

Acknowledgments

Note added in proof

Barnhart and Mellon (1994) have also reported that SF-1 is the GSEB (Barnhart et al., *Endocrinol.* 8: 878–885).

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