

Characterization of the Mouse *FTZ-F1* Gene, Which Encodes a Key Regulator of Steroid Hydroxylase Gene Expression

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The cytochrome P450 steroid hydroxylases are coordinately regulated by steroidogenic factor 1 (SF-1), a protein expressed selectively in steroidogenic cells. Based on its expression in steroidogenic tissues and DNA-binding specificity, we isolated a putative SF-1 cDNA from an adrenocortical cDNA library. As evidence that this cDNA encodes SF-1, we now show that it is selectively expressed in steroidogenic cells, that an antiserum against its protein product specifically abolishes the SF-1-related gel-shift complex, and that its coexpression increases promoter activity of the 21-hydroxylase 5'-flanking region in transfection experiments.

Sequence analyses of the SF-1 cDNA revealed that it is the mouse homolog of *fushi tarazu* factor 1 (FTZ-F1), a nuclear receptor that regulates the *fushi tarazu* homeobox gene in *Drosophila*. A second FTZ-F1 homolog, embryonal long terminal repeat-binding protein (ELP), was recently isolated from embryonal carcinoma cells. SF-1 and ELP cDNAs are virtually identical for 1017 base pairs, including putative DNA-binding domains, but diverge at their 5'- and 3'-ends. One genomic clone contained both SF-1- and ELP-specific sequences, confirming their origin from a single gene. Characterization of this gene defined shared exons encoding common regions and alternative promoters and 3'-exons leading to differences between the two FTZ-F1 transcripts.

We used *in situ* hybridization with transcript-specific probes to study the ontogeny of SF-1 and ELP expression. ELP transcripts were not detected from embryonic day 8 to adult, consistent with its previous isolation from embryonal carcinoma cells and its postulated role in early embryonic development. In contrast, SF-1 transcripts were first detected at embryonic day 12, when steroidogenic organs begin to develop and steroidogenic enzymes are first expressed, and persisted thereafter at high levels in

adrenal glands and testis. Collectively, these studies establish that the mouse homolog of the *FTZ-F1* gene generates developmentally regulated transcripts, one of which encodes an essential regulator of steroid hydroxylase gene expression. (*Molecular Endocrinology* 7: 852-860, 1993)

INTRODUCTION

The regulated production of steroid hormones requires a related group of cytochrome P450 steroid hydroxylases that convert cholesterol to the various steroid products (reviewed in Ref. 1). We recently isolated an orphan nuclear receptor, designated steroidogenic factor 1 (SF-1), which we proposed regulates the coordinate expression of three mouse steroid hydroxylases by interacting with a shared promoter element (2). The putative SF-1 cDNA closely resembles a cDNA isolated from ECA2 embryonal carcinoma cells, designated embryonal long terminal repeat-binding protein (ELP) (3); both sequences are identical for approximately 1000 base pairs (bp), including their zinc finger DNA-binding domains, but diverge at their 5'- and 3'-ends.

Sequence comparisons further indicated that the SF-1/ELP transcripts are homologs of an orphan receptor, designated FTZ-F1, that regulates the expression of the *Drosophila fushi tarazu* (*ftz*) homeobox gene (4, 5). Interestingly, gel mobility shift assays had discriminated two forms of the *Drosophila* FTZ-F1 protein with identical or closely related DNA recognition specificities: an early form, which correlates with *ftz* expression, and a late form, which follows *ftz* expression and serves an unknown function (4).

In this report, we verify that the putative SF-1 cDNA actually encodes the shared regulator of steroid hydroxylase gene expression, explore the relationship between the SF-1 and ELP transcripts, and define their sites of expression. Our results suggest that developmentally regulated products of the *FTZ-F1* gene play

differential roles in early embryonic development and in maintaining the steroidogenic capacity of steroidogenic tissues.

RESULTS

The Putative SF-1 cDNA Encodes a Regulator of Steroid Hydroxylase Gene Expression

We originally isolated a putative SF-1-encoding cDNA by virtue of the homology between its DNA-binding domain and that of another orphan nuclear receptor, retinoid X receptor- β . As defined by Northern blotting analyses, this cDNA was only expressed in steroidogenic tissues, where it encoded a protein that bound steroidogenic regulatory elements with a recognition specificity indistinguishable from authentic SF-1 (2). These studies, however, did not absolutely exclude the possibility that this cDNA encoded another DNA-binding protein with the same recognition specificity as SF-1.

To study more rigorously the link between this cDNA and SF-1, we first performed *in situ* hybridization assays to identify the cells in steroidogenic tissues that expressed transcripts. As shown in Fig. 1, high levels of SF-1-specific transcripts were detected in three classical steroidogenic cells: adrenocortical cells, testicular Leydig cells, and theca cells of the ovary. Additional analyses of ovary showed that SF-1 was also expressed at high levels in corpus luteum (Ikeda, Y., unpublished observation), consistent with our previous demonstration of SF-1 in corpus luteum RNA in Northern blotting analyses (2). Lower levels of SF-1 transcripts were present in ovarian granulosa cells (Fig. 1, lower panel). Finally, although the significance of this finding remains to be determined, the SF-1-specific antisense probe also produced signals in some seminiferous tubules (see Fig. 1, middle panel). Control hybridizations with an SF-1-specific sense probe showed no hybridization above background in any of these cell types (Ikeda, Y., unpublished observation). These results significantly extend previous Northern blotting analyses of SF-1 expression, directly linking expression of this putative SF-1-encoding cDNA and steroidogenic cell types *in vivo*.

Next, we examined the effect in gel mobility shift assays of polyclonal antisera raised against the amino-terminal region of the protein encoded by this cDNA (see *Materials and Methods* for a description of the sequences used for immunization). As previously reported (6), Y1 adrenocortical nuclear extracts formed prominent, SF-1-related shift complexes with both the aldosterone synthase -310 and 21-hydroxylase -65 elements (complex I in Fig. 2). Addition of antisera raised against the product of the putative SF-1 cDNA abolished this complex; in contrast, none of the other shift complexes seen with the 21-hydroxylase -65 element were affected by the antisera. Although incubation of both probes with control and immune sera also led to the appearance of larger complexes whose

precise nature remains to be defined, the specific effect of the antisera on the SF-1-related complex strongly supports the model that this cDNA encodes SF-1.

Finally, we analyzed the ability of the putative SF-1 cDNA to affect transcriptional activity. First, we determined the effect of coexpression of the SF-1 transcript on expression of p21-OHaseGH, a reporter plasmid that contains 6.4 kilobases of 5'-flanking sequences from the mouse 21-hydroxylase gene upstream of the human GH (hGH) reporter gene (7). As shown in Fig. 3, cotransfection of mouse Y1 adrenocortical cells with the putative SF-1 cDNA increased by 12-fold the expression of the hGH reporter gene. Supporting the specificity of this effect, no enhancement followed cotransfection with an expression plasmid driving the expression of the ELP cDNA, which has an identical DNA-binding domain (Fig. 3).

To establish the role of SF-1 sites in this induction, we examined the effect of SF-1 coexpression on a reporter plasmid (p-65-luc) containing five copies of a known SF-1-binding site cloned upstream of the core promoter from PRL driving the expression of the luciferase reporter gene (7). As shown in Fig. 4, the parental plasmid pPro36-luc was expressed at low levels and showed no response upon cotransfection with SF-1. In contrast, p-65-luc, which has five copies of the 21-hydroxylase -65 element, showed a marked response to SF-1, with a 50-fold increase in luciferase activity. The finding that expression of the putative SF-1 cDNA markedly augments promoter activity of the 21-hydroxylase 5'-flanking region and of a reporter plasmid containing a known SF-1-responsive element strongly supports the link between this cDNA and SF-1.

A Single Structural Gene Encodes Both SF-1 and ELP

Based on genomic blotting experiments and partial sequence of the SF-1 cDNA, we proposed that the SF-1 and ELP transcripts arise from a single structural gene by alternative promoter usage and/or RNA splicing (2). We therefore screened a Balb/c mouse genomic library in the EMBL 3 bacteriophage vector with 5'- and 3'-probes derived from our SF-1 cDNA and isolated overlapping clones containing both 5'- and 3'-sequences (see *Materials and Methods* for the derivation of the probes). Hybridization with ELP-specific probes showed that the SF-1 clones also contained sequences specific for the ELP transcript, establishing definitively that both cDNAs derive from a single structural gene, which we designate *FTZ-F1* in keeping with its original identification in *Drosophila* (5).

We next determined the organization and DNA sequence of the gene encoding the SF-1 and ELP transcripts. Probes corresponding to the unique 5'-ends of each cDNA were used to isolate the 5'-ends of the transcription units; the transcription start site for SF-1 in Y1 cells was identified by primer extension and RNase protection assays and by comparison with cDNAs isolated by 5'-rapid amplification of cDNA ends

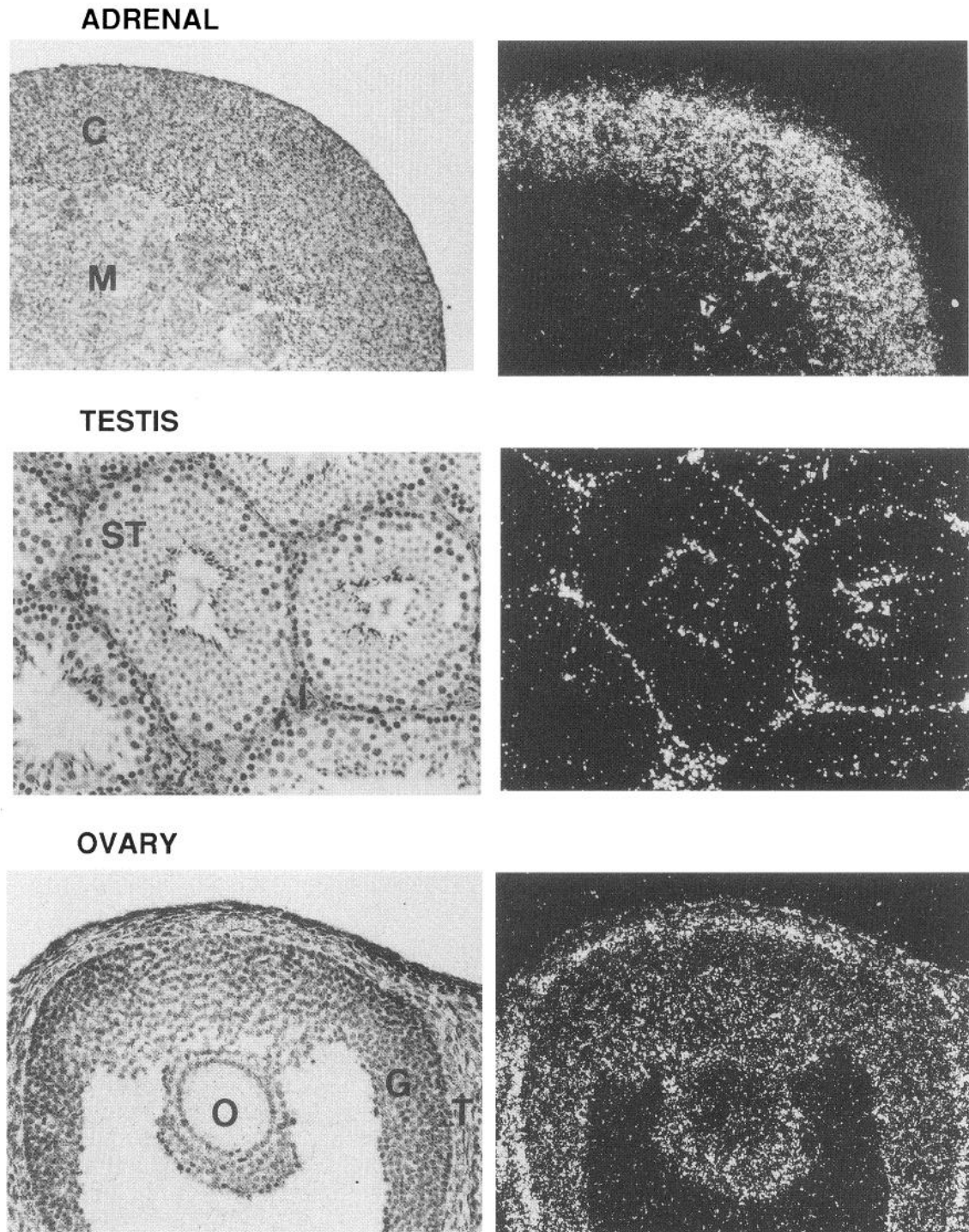


Fig. 1. SF-1 Is Selectively Expressed in Steroidogenic Cell Types

The patterns of expression of transcripts encoded by the putative SF-1 cDNA were assessed by *in situ* hybridization analyses with cRNA probes as described in *Materials and Methods*. Bright-field examination is shown on the *left* and dark-field examination on the *right*. Adrenal gland (magnification, $\times 92.5$): C, cortex; M, medulla. Testis (magnification, $\times 185$): ST, seminiferous tubule; I, interstitium. Ovary (magnification, $\times 185$): O, oocyte; G, granulosa cells; T, theca cells.

(8). Although the precise transcription start site of ELP has not been defined, primer extension analyses suggest that transcription initiates near the most 5'-sequences contained in the previously reported ELP cDNA (3; Kim, E., unpublished observation). Exon-in-

tron junctions were identified by comparison of genomic and cDNA sequences; in all cases (Fig. 5), canonical GT.....AG splice donor/acceptor sequences were present at exon-intron junctions (9).

As listed in Fig. 5 and summarized schematically in

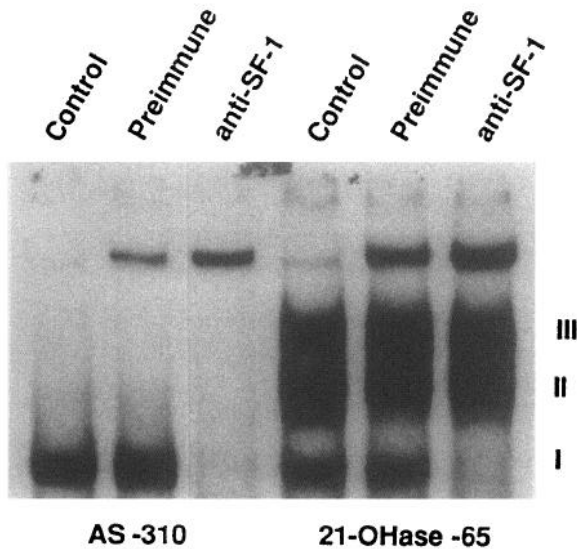


Fig. 2. Antisera against the Putative SF-1 cDNA Specifically Inhibit the SF-1-Related Gel Shift Complex

Rabbit polyclonal antibodies against the amino terminal region of the putative SF-1 cDNA were produced as described in *Materials and Methods*. Gel mobility shifts were performed with the aldosterone synthase -310 (AS -310) and 21-hydroxylase -65 (21-OHase -65) elements and nuclear extracts from Y1 adrenocortical cells. Where indicated, the nuclear extract was preincubated with either preimmune or immune sera before the addition of probe.

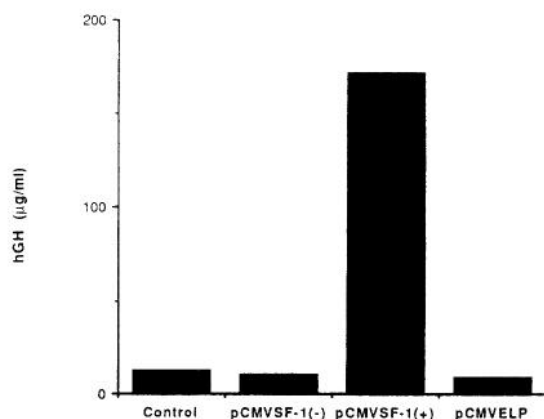


Fig. 3. The Putative SF-1 cDNA Augments Promoter Activity of the 21-Hydroxylase Promoter Region in Transfected Y1 Adrenocortical Cells

Y1 adrenocortical cells were transfected with 2.5 μ g p21-OHaseGH, and hGH was measured as described in *Materials and Methods*. Where indicated, cells were cotransfected with 5 μ g pCMV expression vector directing the expression of the putative SF-1 cDNA in either the correct (+) or reverse (-) orientation or the ELP cDNA in the correct orientation. The results are the mean of four independent transfections for each condition.

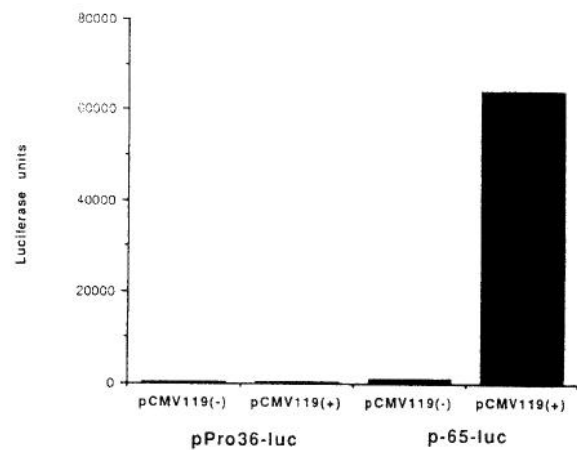


Fig. 4. Coexpression of the Putative SF-1 cDNA Increases Luciferase Expression in a Manner Dependent on the 21-hydroxylase-65 SF-1 Site

Y1 adrenocortical cells were transfected with 2.5 μ g pPro36-luc or with p-65-luc, lysed 48 h after transfection, and luciferase expression was measured using a kit from Promega as described in *Materials and Methods*. Cells were cotransfected with 5 μ g SF-1 expression vectors in the correct (+) or reverse (-) orientations. The results are the means of four independent transfections for each condition.

Fig. 6, the complete SF-1 coding sequence predicts a protein with an *M_r* of approximately 52,000, consistent with previous estimates of the size of SF-1 (2). SF-1 is transcribed from the more 5' promoter, and its entire first exon contains 5'-untranslated sequences. Although we have not definitively mapped the ELP transcription initiation site, the most 5' sequences in the ELP cDNA (3) fall within the first intron of SF-1 (position 228 in Fig. 5). Splicing at the 3'-splice site of the SF-1 first intron generates common ELP and SF-1 coding sequences just upstream of sequences encoding the first zinc finger. SF-1 translation initiates at an internal Met in the ELP sequence, and only 12 residues precede the shared first zinc finger region (positions 694-760). The next two shared exons encode the second zinc finger region (positions 908-1039) and a conserved region, termed the FTZ-F1 box, recently implicated in DNA binding (positions 1122-1214) (10).

After additional shared sequences that encode part of the ligand-binding domain (11), alternative splicing generates ELP-specific sequences. In contrast, two additional exons generate the 3'-SF-1-specific sequences. The precise 3'-terminus of the ELP gene has not been mapped, as a full-length ELP cDNA has not been isolated, and sequencing of genomic clones has not identified a potential AATAAA 3'-splice signal. Nonetheless, as shown in Figs. 5 and 6, the entire organization of SF-1 and the general features of the *FTZ-F1* gene that lead to the similarities and differences between the ELP and SF-1 transcripts have been elucidated.

GTCCCGCTCCGCCCGCTGCCCGCCGTCTGGTGAAGAAGTTCTGAGAGCCGCTAGCCCTACCTGAGGCC TGGGAGCC TCCCCACAGDACCCTGGTGTCCAGTGTCCAC 121

CCTTATCCGGCTGAGAAATTCCTCTCCGTTCAGTAAGTGAAGCCGGGGCCAGGCTCTANNNNNNNNNAATGTCTGTGGCCACCGCATTCCTGTCCCTGTCCACCGAGTATCCCTCTCCGT 242

→ 3.1 kb

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CCCRCGTCCGACGTATCTGATTTCTCAGAATCGGGTTTGTCTCAGACAAAGAAATCTGGATGAAATGCAATCGAATCCGAGGGTCCCGGATCGGGCCGGCAGAGCGCGGAGAA 484

Met

GCAGCCCTGGAACCGCGCGGTGGCTGAGCTGCAGCGCAGGGACATGGCCCAAAACCCCGCCCGCCCGGCTGGGGACCGCCCGTGTGACAGACCAAGGCCAAATCCCAAGCCAGT 605

CCCCGTCGGCCCGCGTGAACCGATCCTCTCCACAGCGCCGACCGCGGCATGGACTACTCGTACGACTGAGGACTGGACGAGCTGTGCTCAGTGTGGTGCAGAAGGTGTCCGGCTA 726

Met Asp Tyr Ser Tyr Asp Glu Asp Leu Asp Glu Leu Cys Pro Val Cys Gly Asp Lys Val Ser Gly Tyr

CCACTACGGGCTGCTCACGTGGAGAGCTGCAAGGTGTGGGCTACCACATAGGGCTGCTCAGTGGGAGCTGCAAGGTGAGCGCAAGCGGGAGCGGAGGGTGTAGGCACCGGGTGG 847

His Tyr Gly Leu Leu Thr Cys Glu Ser Cys Lys

CAGCGTGGCGCGGCAAGGATGGGACTGGCGCTCAATCCCTTCTCTGGAGAGGCTTCTCAAGCGCAGCTCCAGAACAAAGCATTACAGTGACCCAGGAGTCCAGAGC 968

Gly Phe Phe Lys Arg Thr Val Glu Asn Asn Lys His Tyr Thr Cys Thr Glu Ser Glu Ser

TGCAAAATCGACAAGCGCAGCTAAGCGCTGTCCTTCTCGCGCTCCAGAAAGTGCCTGACGCTGCGCTGCAAGTGGCGTCTGCAAGTGGGCTGCTGCAAGCGCGGAGCCCTGCATGCTGGCC 1089

Cys Lys Ile Asp Lys Thr Glu Arg Lys Arg Cys Pro Phe Cys Arg Phe Glu Lys Leu Thr Val Gly Met Arg Leu Glu

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2.0 kb

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Gln Ile Arg Ala Asn Gly Phe Lys Leu Glu Thr Gly Pro Pro Met Gly Val Pro Pro Pro Pro Pro Pro Pro Asp Tyr Met Leu Pro Pro Ser Leu His Ala Pro Glu Pro Lys Ala

CTGGCTCTGGCCCGCCAGTGGGCGCTGGGTGACTTTQAGCCCAATCTTACCAGTGGCTGGCTGGTCCCGCCAGCCTGTGGCTATCTATCTCCCTCTCTAACCGCA 1452

Leu Val Ser Gly Pro Pro Ser Gly Pro Lys Glu Arg Phe Gly Ala Pro Ser Leu Pro Met Ala Val Pro Gly Pro His Gly Pro Leu Ala Gly Tyr Leu Tyr Pro Ala Phe Ser Asn Arg

CCATCAAGCTGAGTATCCAGGCCCTATGCCAGCCCGCCAGCCAGCCAGCCAGCCAGCCATCCAGACTATCCAGAGCCCTTCCAGGAGGGCCCAATGACAGAGCTCATATTGACGCTGT 1573

Thr Ile Lys Ser Glu Tyr Pro Glu Pro Tyr Ala Ser Pro Pro Glu Pro Gly Pro Tyr Ser Tyr Pro Glu Pro Phe Ser Gly Gly Pro Asn Val Pro Glu Leu Ile Leu Glu Leu

GCAACTAGAGCCAGAGGAGCAGCTGGCGCTGCATCGTGGCTGTCTCAGGAGCCAGCCAAAAGCGCTGACCCAGCCAGGCCCTTCAGCTCTCTGCAGAAATGCCGACGAC 1694

Gln Leu Glu Pro Glu Glu Asp Gln Val Arg Ala Arg Ile Val Cys Leu Glu Glu Pro Ala Lys Ser Arg Ser Asp Glu Pro Ala Pro Phe Ser Leu Leu Cys Arg Met Ala Asp Gln

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5.8 kb

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Asn Cys Trp Ser Glu Leu Leu Val Leu Asp His Ile Tyr Arg Gln Val Gln Tyr Gly Lys Glu Asp Ser Ile Leu Leu Val Thr Gln Gln Gln

CTGCAATAACCGCCCTTCCAGGCTGACTGGGACACCCCAAATTCCAAATTCAGGACATGCATAGCAAGCTTCTCTGTCCTCGGGCCATITGAGGAGCCACAGTGGAAATG 2057

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0.9 kb

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2.5 kb

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Asp Val Lys Phe Leu Asn Asn His

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Ala Leu Ser Met Gln Ala Lys Glu Tyr Leu Tyr His Lys His Leu Gly Asn Glu Met Pro Arg Asn Leu Leu Ile Glu Met Leu Gln Ala Lys Gln Thr

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ATTTGTAATTTGCATCTGAATCGAG 4604

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Fig. 5. Sequence of the Mouse FTZ-F1 Gene

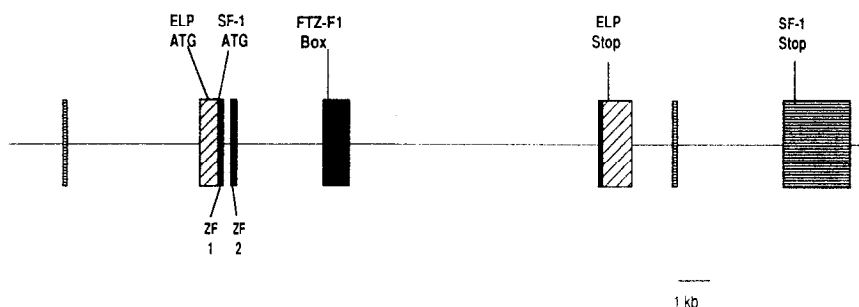


Fig. 6. Organization of the Mouse *FTZ-F1* Gene

The schematic organization of the mouse *FTZ-F1* gene is drawn to scale. *Horizontal markings* designate SF-1-specific sequences, *diagonal cross-hatchings* designate ELP-specific sequences, and *black shading* designates shared sequences. Indicated positions represent the initiator Met and termination codons for the SF-1 and ELP transcripts. ZF1, the first zinc finger region; ZF2, the second zinc finger region; FTZ-F1, FTZ-F1 box (10).

The Two Transcripts Encoded by the Mouse *FTZ-F1* Gene Have Different Patterns of Expression

Because studies with FTZ-F1 in *Drosophila* suggested developmentally regulated expression of the distinct forms, we studied the ontogeny of ELP and SF-1 transcripts. To this end, we performed *in situ* hybridization analyses with ELP- and SF-1-specific cRNA probes. ELP transcripts were not detected in any mouse developmental stages studied (embryonic day 8 to adult; Ikeda Y., unpublished observation), consistent with the previous isolation of ELP from embryonal carcinoma cells and its postulated role in early embryonic development. In contrast, SF-1 transcripts were first detected in mouse adrenal gland at embryonic day 12 (Fig. 7) and persisted thereafter at high levels. SF-1 expression in the testis likewise began at embryonic day 12 and persisted at high levels throughout the rest of the prenatal period (Ikeda, Y., unpublished observation). Of interest, expression of the cholesterol side-chain cleavage enzyme was first detected in the developing adrenal gland at embryonic day 12 (12), consistent with the model that SF-1 expression is tightly coupled to that of the steroid hydroxylases. Consistent with the relatively inactive state of the fetal ovary, SF-1 expression was not detected in this organ in fetal sections (Ikeda, Y., unpublished observation).

DISCUSSION

In this report, we establish that the mouse homolog of *FTZ-F1* encodes two distinct transcripts: a protein originally isolated from embryonal carcinoma cells, termed

ELP, and a global regulator of steroid hydroxylase gene expression, termed SF-1. We further document that the cloned SF-1 cDNA augments promoter activity of the 5'-flanking region of steroid 21-hydroxylase and that SF-1 is expressed very early in the development of primary steroidogenic tissues, consistent with its postulated key role in the function of steroidogenic cells.

To date, direct demonstration of functional effects of SF-1 has only been obtained with adrenocortical cells. However, SF-1 binding sites are found upstream of all steroidogenic cytochrome P450 genes characterized (13). Experiments with aromatase expression in rat granulosa cells implicate SF-1 in steroid hydroxylase expression in nonadrenocortical steroidogenic cells (14, 15). Moreover, preliminary experiments suggest that SF-1 also regulates Leydig cell expression of oxytocin, a noncytochrome P450 gene (Parker, K., and R. Ivell, unpublished observation), and our *in situ* hybridization experiments (Fig. 1) suggest that SF-1 may be expressed in cells of the germ cell lineage. The emerging evidence thus points to an important role of SF-1 in proper function of all of the primary steroidogenic tissues.

The role of ligand(s) in SF-1-mediated transcriptional activation remains to be defined. Our transfection experiments (Figs. 3 and 4) show significant effects of coexpression of SF-1 in Y1 adrenocortical cells without the addition of any exogenous ligand. As these cells lack endogenous expression of steroid 21-hydroxylase and thus cannot produce either mineralocorticoids or glucocorticoids, it is unlikely that corticosteroid end products subservise such a role. Other candidate ligands include intermediates in the steroid biosynthetic pathway and vitamin E, which is found at very high levels in the adrenal cortex (16). Alternatively, it remains possible

The DNA sequence of the mouse *FTZ-F1* gene and predicted translation of the SF-1 transcript are indicated. All positions are relative to the SF-1 transcription initiation site. The *numbers below the brackets* indicate the sizes of introns whose sequences are not included. Sequences common to both SF-1 and ELP transcripts are underlined with *wide arrows*, sequences that are uniquely found in the SF-1 transcript are underlined with *arrows of intermediate width*, and sequences that are uniquely found in the ELP transcript are underlined with *thin arrows*. The Met at position 450 denotes the initiator Met for ELP.

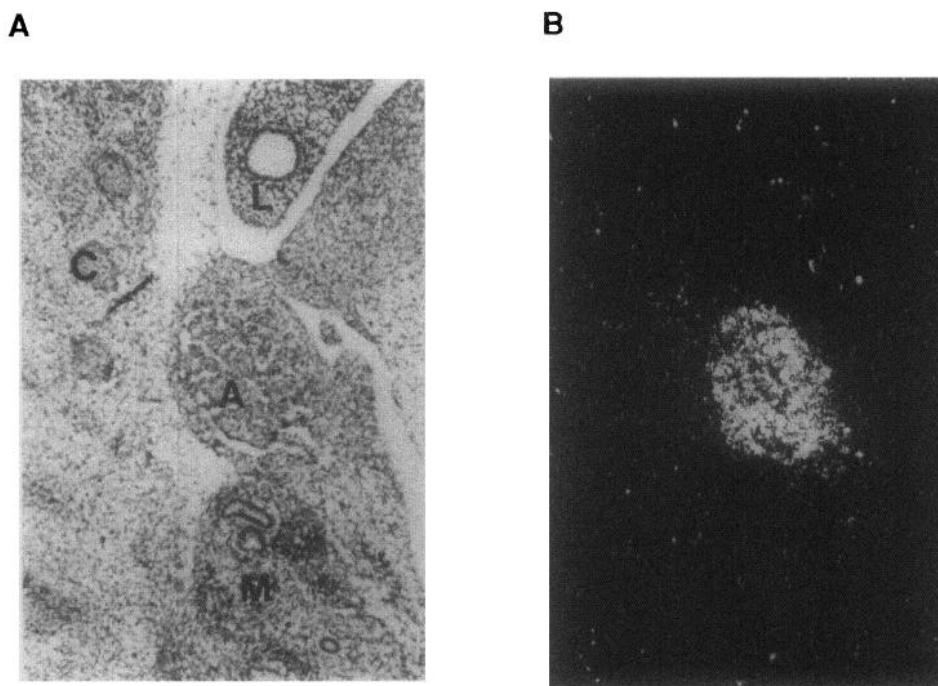


Fig. 7. Expression of SF-1 Is Detected at Early Stages of Adrenal Development

Mouse embryos were obtained from timed pregnant mice at the indicated ages and prepared for *in situ* analyses with the SF-1 cRNA probe as described in *Materials and Methods*. The results from a section that included the adrenal gland are shown (magnification, $\times 92.5$). *Left*, bright-field exam; *Right*, dark-field exam. A, Adrenal primordium; C, cartilaginous precursor; M, metanephros; L, lung.

that SF-1 does not require a specific ligand for activation. NGFI-B, another orphan receptor that regulates steroid hydroxylases (7), apparently activates transcription in a ligand-independent manner.

Recent studies have shown surprising similarities between the nuclear receptors of *Drosophila* and mammals (reviewed in Ref. 17). Among the conserved members are chicken ovalbumin upstream promoter transcription factor/seven-up (18, 19), retinoid X receptor- β /ultraspiracle (20, 21), and hepatocyte nuclear factor 4/tailess (22, 23). Based on these similarities, it is not entirely unexpected that mice express a homolog, which we designate *FTZ-F1* in keeping with its original description in *Drosophila*. Based on the strict maintenance of developmentally regulated forms of FTZ-F1, certain hypotheses regarding the function of the related transcripts in the two species seem attractive. The early form of *Drosophila* FTZ-F1 is proposed to regulate the expression of the *ftz* homeobox gene. Although the corresponding gene in mice remains to be identified, it is plausible that ELP (the early form of FTZ-F1 in mice) regulates the expression of a mouse *hox* gene that contributes to embryological development. Clearly, ELP does not regulate the expression of the steroid hydroxylases, as an ELP expression plasmid did not affect 21-hydroxylase promoter activity under the conditions where a marked response to SF-1 was obtained (see Fig. 3).

Models can likewise be formed about the late form

of FTZ-F1 in *Drosophila*. There are a number of similarities between the vertebrate steroid hormones and the ecdysteroids of invertebrates. In particular, both ecdysone and mammalian steroids are derived from cholesterol by the action of cytochrome P450 mixed-function oxidases (1, 24), and both act by binding to ligand-responsive nuclear receptors (25, 26). Finally, both compounds play key roles in the normal development and sexual function of the organism. It is tempting to speculate that the late form of FTZ-F1, for which no function has been elucidated, regulates the expression of enzymes involved in ecdysone biosynthesis. Ultimately, proof of this model will require the characterization and cloning of the proteins that synthesize ecdysone and the demonstration that late FTZ-F1 regulates their expression.

MATERIALS AND METHODS

Materials

Restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer-Mannheim (Indianapolis, IN). Radionuclides were purchased from New England Nuclear/E. I. DuPont (Boston, MA). Levels of hGH were measured by RIA using a kit purchased from Nichols Diagnostics. Expression of luciferase was measured with an LKB (Piscataway, NJ) luminometer using an assay kit purchased from Promega (Madison, WI). Sequenase II kits for sequence analysis of double-stranded plasmids were purchased from

United States Biomedical (Cleveland, OH). A kit for 5' rapid amplification of cDNA ends was purchased from Bethesda Research Laboratories (Gaithersburg, MD).

Preparation of Anti-SF-1 Antisera

Sequences comprising the putative zinc finger DNA-binding domain of SF-1/ELP (exonic sequences corresponding to positions 136–1218 in the *FTZ-F1* sequence in Fig. 5) were cloned in the prokaryotic expression plasmid pGEX-1 λ t and expressed in *Escherichia coli* as previously described (2). The crude bacterial lysate containing the glutathione S-transferase-SF-1 fusion protein was partially purified by affinity chromatography as described (27). Briefly, 12 ml bacterial extract was mixed with 500 μ l of a 1:1 suspension of glutathione (GSH)-sepharose beads and PBS and gently rocked at 4 C for 30 min. The GSH-sepharose beads were centrifuged for 5 min at 1000 \times *g* and washed three times with 5 ml of PBS + 10 mM EDTA. The protein was then resuspended in 2 ml PBS + 10 mM EDTA at a concentration of approximately 200 μ g protein/ml and stored at 4 C. Rabbits at Organon-Teknica-Cappel laboratories (West Chester, PA) were then immunized with the affinity-purified SF-1-GST/GSH-sepharose slurry as described (27).

Cell Culture

Mouse Y1 adrenocortical tumor cells were cultured and transfected by the CaPO₄ precipitation technique as described (7), using 2.5 μ g of the reporter gene and 5 μ g of the SF-1 expression plasmid. Levels of hGH were determined by RIA 48 h after transfection. In experiments with the firefly luciferase reporter plasmid, cells were lysed and luciferase activity determined according to the protocol supplied with the luciferase assay kit.

Plasmids

The plasmids used in this study included: p21-OHaseGH, which contains 6.4 kilobases of 5'-flanking sequences from the mouse 21-hydroxylase gene placed upstream of an hGH reporter gene (7); pCMV-SF-1(+), a cytomegalovirus (CMV) promoter-based eukaryotic expression plasmid, which contains SF-1 cDNA sequences from the *EcoRI* site 33 bp upstream of the initiator Met to 604 bp downstream of the termination codon; pCMV-SF-1(-), a CMV promoter-based eukaryotic expression plasmid that contains the identical SF-1 sequences in the reverse orientation; pCMV-ELP, a CMV promoter-based eukaryotic expression plasmid that contains the ELP coding sequences in the correct orientation; pSF-1 5', which contains SF-1 cDNA sequences from +30 to +114; pSF-1 3', which contains SF-1 3'-untranslated sequences (3488–3733 in Fig. 5); pELP 5', which contains ELP sequences from +1 to +430 (3); pPro36-luc (28), which contains the core promoter from the PRL gene upstream of the firefly luciferase reporter gene; and p-65-luc, a derivative of pPro36-luc with five copies of the 21-hydroxylase -65 element inserted upstream of the PRL promoter (17).

Gel Mobility Shift Assays

Crude nuclear extracts were prepared from Y1 cells by the method of Shapiro *et al.* (29). Gel mobility shift assays with the aldosterone synthase -310 and 21-hydroxylase -65 probes were performed as described (2). Where indicated, preimmune or immune anti-SF-1 antiserum was included in the binding reaction for 30 min before addition of probe in the gel shift assays.

In Situ Hybridization Analyses

Seven-micrometer-thick paraffin sections of mouse adult tissue (adrenal, testis, and ovary) and embryos (E8–E16) were

purchased from Novagen (Madison, WI). *In situ* hybridizations with [³⁵S]cRNA probes were performed using an *in situ* hybridization kit obtained from Novagen according to the recommended protocol. Probes included SF-1 3', which contains sequences from the SF-1 3'-untranslated region (positions 3488–3733 in Fig. 5), and ELP 5', which contains sequences from the 5'-untranslated region of ELP (+1 to +430 in Ref. 3). Exposures were for 3–4 weeks, and slides were counterstained with methyl green. No signals over background were seen with any of the sense probes.

Note Added in Proof

After this paper was submitted, Honda *et al.* reported the sequence of a cDNA encoding Ad4-binding protein, the bovine homolog of SF-1 (Honda S.-I. *et al.*, *J Biol Chem* 268:7494–7502). They also concluded that their protein was the bovine homolog of *FTZ-F1*.

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