MULTIPLE APPROACES TO THE STUDY OF STEROIDOGENIC FACTOR 1:
IDENTIFICATION OF A NOVEL REGULATORY ELEMENT AND
IDENTIFICATION OF NOVEL TARGET GENES

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To Joe

"To love and be loved is to feel the sun from both sides"

David Viscott

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by

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DISSERTATION

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Publication No. _____

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Steroidogenic Factor 1 (SF-1) is an essential component of the hypothalamic-pituitary-adrenal-gonadal axis. SF-1 knockout (KO) mice lack adrenals, gonads, the ventromedial hypothalamic nucleus (VMH), and pituitary gonadotropes. SF-1 is a transcription factor implicated in the regulation of many genes important in endocrine function. Research into the regulation of SF-1 expression, mostly focused on the proximal promoter, has been unable to fully explain the expression pattern of SF-1. I used DNase hypersensitivity mapping to search for novel regulatory regions of the SF-1 genomic region. One region between the 6th and 7th exons of SF-1 had tissue specific DNase I hypersensitivity. Analysis of this region revealed high conservation

with the human genomic sequence and a smaller region that was also highly conserved in the chicken genomic sequence. Transient transfection assays and electrophoretic mobility shift assays have been employed to investigate this conserved element for enhancer activity.

Numerous genes are target genes of SF-1, yet loss of known target genes fail to explain why the adrenals, gonads and VMH fail to develop in SF-1 KO mice. I used an SF-1/eGFP transgene as a reporter in both WT and KO E16.5 embryos. eGFP+ cells from the developing VMH of these mice were collected through the use of FACS. Several potential target genes of SF-1 have been identified and analysis of these genes is an ongoing project.

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ABBREVIATIONS

Ad4BP, adrenal 4 binding protein
BAC, Bacterial artificial chromosome;
BDNF, brain-derived neurotrophic factor
Cbln1, Cerebellin
CNS-specific, central nervous system specific knockout
CYP11A, cholesterol side chain cleavage, SCC
CYP11b1, steroid-11-beta hydroxylase
CYP21, 21-hydroxylase
Dab2, disabled 2
Dax-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome
eGFP, enhanced green fluorescent protein
ELP, embryonal long terminal repeat-binding protein
EMSA, electrophoretic mobility shift assay
FACS, fluorescence-activated cell sorting
Foxg1, forkhead box g1, brain factor 1 (Bf-1)
FSH, follicle stimulating hormone
GCNF, germ cell nuclear factor
KO, knockout
LH, lutanizing hormone
LRH-1, liver receptor homolog 1
Nr0B1, nuclear receptor subfamily 0, group B, member 1, Dax-1
Nr5a1, nuclear receptor subfamily 5, group A, member 1; SF-1
Nr5a2, nuclear receptor subfamily 5, group A, member 2; LRH-1
QRT-PCR, quantitative reverse transcription polymerase chain reaction
SCC, side chain cleavage, CYP11A1
SF-1, steroidogenic factor 1
Shox2, short stature homeobox protein, Prx-3
SRB1, scavenger receptor, class B, type 1
SRY, sex determining region Y
StAR, steroidogenic acute regulatory protein
Ten-C, tenascin c
Ten-M2, Odd oz, odz2
VMH, ventromedial hypothalamic nucleus.
WT, wild type

Chapter 1 Introduction to Steroidogenic Factor 1

1.1 Identification of Steroidogenic Factor 1

A Common Element in the Promoter of Several Cytochrome P450 Genes

SF-1 was first identified as a transcription factor that bound to shared elements in several of the cytochrome P450 genes involved in steroidogenesis (Bogerd et al., 1990; Rice et al., 1990; Rice et al., 1990; Rice et al., 1991). Through the use of transient transfections in steroidogenic and non-steroidogenic cell lines and DNase I footprinting assays, a protein present in only the steroidogenic cell lines that bound to a common AGGTCA element was identified in the promoters of the 21-hydroxylase (Cyp21) (Rice et al., 1990), side chain cleavage (SCC ;CYP11A1) (Rice et al., 1990), and steroid-11-beta hydroxylase (CYP11b1) (Bogerd et al., 1990) genes. Cloning of the protein that bound this element, named Steroidogenic Factor 1 (SF-1) or Adrenal 4 binding protein (Ad4BP) revealed that it was the mouse homolog of Drosophila Ftz-F1 and confirmed the ability of this protein to bind to the promoters of the P450 genes (Lala et al., 1992; Morohashi et al., 1992).

Genomic organization of the Nr5a1 locus

In the mouse and human, SF-1 is encoded by the SF-1 gene (Nr5a1 in mouse and NR5A1 in human, previously known as the Ftz-F1 gene) (Ikeda et al., 1993), which contains 7 exons (Ninomiya et al., 1995) (Figure 1-1). There are potentially 4 transcripts produced from the mouse Nr5a1 locus. The other transcripts, termed ELP1, ELP2, and ELP3 are discussed below. The SF-1

transcript uses 7 exons. Exon 1 is a 5' untranslated region and exon 7 contains a large 3' untranslated region. Exon 3 contains the zinc finger, exon 4 contains the A box, and the exon 7 contains the AF-2 domain. These protein domains and their functions are discussed below in a description of the SF-1 protein.

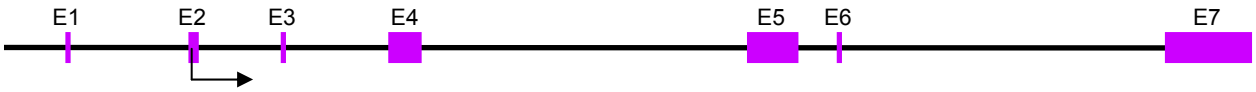


Figure 1-1Genomic Organization of Nr5a1.
The boxes indicate the seven exons of SF-1 produced from the Nr5a1 locus. This organization of introns and exons is conserved between mouse and human. The arrow indicates the translation start of SF-1. Exon 1 is a 5' untranslated region and exon 7 contains a 3' untranslated region.

Mouse and human chromosomes have a conserved gene arrangement

Mouse SF-1 maps to chromosome 2 and human SF-1 maps to 9q33 (Taketo et al., 1995). The intron/exon boundaries are conserved between mouse and human (Oba et al., 1996). The nuclear receptor, Nr6a1 (germ cell nuclear factor, GCNF) is found 5' to Nr5a1 and GPR144 is found in the 3' direction (NCBI, 2004) (Figure 1-2). This gene arrangement is maintained in mouse and human genomic DNA. GPR144 is an adhesion G-protein-coupled receptor which is a membrane-bound protein with long N-terminus containing multiple domains (Bjarnadottir et al., 2004). ESTs for GPR144 have a widespread tissue distribution (Bjarnadottir et al., 2004). GCNF is an orphan member of the nuclear receptor super family, which is distantly related to other members of the superfamily and forms a distinct family of receptors (Cooney et al., 1998). GCNF is a transcriptional repressor and is expressed in germ cells and the developing mouse nervous system between E8.5 and E9.5 (Agoulnik et al., 1998; Susens et al., 1997). It is not known if there are any regulatory regions common to SF-1, GCNF, and GPR144.

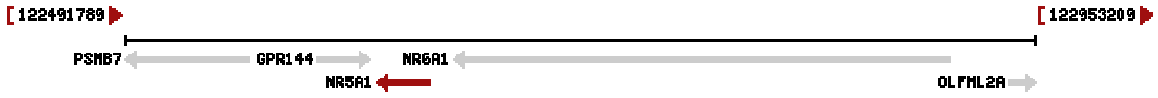


Figure 1-2 Gene Neighbors of Nr5a1 on Mouse Chromosome 2
Chromosome 2 of the mouse contains Nr5a1. The genes surrounding SF-1 are Nr6a1, (Germ cell nuclear factor), a member of the nuclear hormone receptor superfamily and GPR144, a G-protein coupled receptor

Alternative transcripts from the Nr5a1 locus: ELP1, ELP2, ELP3 and SF-1

Four different transcripts reportedly are produced from the mouse Nr5a1 locus. These transcripts vary in both 5' and 3' exons and tissue distribution. ELP1 and ELP2 are not produced at high levels in tissues with SF-1, although they can be detected at varying levels depending on the assay employed (Morohashi et al., 1994; Ninomiya et al., 1995). One of the isoforms, ELP3, has a different 5' end but produces a protein that is identical to SF-1 and likely arises from alternative promoter usage in the pituitary (Ninomiya et al., 1995). ELP1 and ELP2 are unlikely to be expressed in humans and rats due to an in-frame stop codon (Oba et al., 1996). Knockout mice (discussed below) that lack only the ELP3 and SF-1 transcripts recapitulate the phenotype seen in the more general KO (which disrupts the zinc finger common to all four transcripts), proving the ELP1 and ELP2 are not essential transcripts from the Ftz-F1 locus (Luo et al., 1995). The comparison of mouse/human/rat/chicken transcripts together with the KO mice data suggest that ELP1 and ELP2 are not important transcripts and that ELP3 can be considered to be an SF-1 equivalent derived from the utilization of an alternative promoter.

1.2 Expression of SF-1

SF-1 is expressed in a cell-and tissue-selective manner

SF-1 transcripts can be detected in the urogenital ridge at E9.5 (Ikeda et al., 1994), before distinct adrenal and gonadal precursors have emerged. By E11, the gonads and adrenals have separated into distinct populations of cells, both of which express SF-1 (Hatano et al., 1996; Ikeda et al., 1994). SF-1 soon localizes to the adrenal cortex as the chromaffin cells migrate into the adrenal (Ikeda et al., 1994). This expression pattern persists thereafter as SF-1 is expressed in steroidogenic cells of the adrenal cortex and not the adrenal medulla (Ikeda et al., 1993; Morohashi et al., 1994).

As the gonads form, SF-1 is expressed in a cell-specific manner in both the developing testis and ovary. It is at E12.5, that the ovary and the testis can first be distinguished morphologically. The fetal testis expresses SF-1 in both the fetal Sertoli cells and Leydig cells (Ikeda et al., 1994). In pubertal rats, SF-1 was detected in Sertoli and Leydig cells; and thereafter expression of SF-1 in the Sertoli cells decreases, while remaining strong in the adult Leydig cells (Hatano et al., 1994; Ikeda et al., 1993). The ovary does not display the level of morphological differentiation during embryogenesis as the testis. In the fetal ovary, SF-1 expression decreases at E13.5 and does not increase again until E18.5 (Ikeda et al., 1994). In the adult, SF-1 expression can be detected in the ovarian theca and granulosa cells, as well as the corpus luteum (Ikeda et al., 1993; Morohashi et al., 1994).

In the brain, SF-1 can be detected in the developing diencephelon by as early as E9.5 and is clearly expressed in the region that will form the hypothalamus by E14.5 (Ikeda et al., 1994). SF-1 expressing cells form the ventromedial hypothalamus (VMH) and expression of SF-1 is seen in this region throughout development and in adult mice (Ikeda et al., 1995; Ikeda et al., 1994).

Two other sites of SF-1 expression are the pituitary and spleen. At E13.5, SF-1 transcripts can be detected in the developing pituitary, with progressive increases at E14.5 and E17.5 (Ingraham et al., 1994). SF-1 transcripts localize to the anterior pituitary where they are expressed in the gonadotropin producing cells (Ikeda et al., 1995; Ikeda et al., 1994). Expression in the spleen appears at E14.5 and, although it has not been possible to determine the exact cells that express SF-1, there is some suggestion that they are embryonic components of the vascular system (Morohashi et al., 1999). SF-1 is expressed in the endothelial cells of the splenic venous sinuses and pulp vein (Morohashi et al., 1999).

1.3 Characterization of SF-1 KO Mice

Global SF-1 knockout mice fail to develop gonads, adrenals, and the ventromedial hypothalamic nucleus

Three different groups independently generated SF-1 KO mice. The phenotypes of all of the knockouts were essentially identical and are summarized below (Luo et al., 1994; Morohashi and Omura, 1996; Sadovsky et al., 1995). SF-1 KO mice die shortly after birth. Analysis of newborn pups revealed that KO pups are born at the expected Mendelian ratio, showing that there is no embryonic

death of the KO embryos. Gross examination of the SF-1 KO mice showed that SF-1 KO mice lack adrenal glands and gonads and the internal and external genitalia are female, irrespective of genetic sex. Due to the lack of adrenals, the postnatal death is suggested to be due to the lack of corticosteroids, since steroid treatment allowed for the survival of newborn pups (Luo et al., 1994; Morohashi and Omura, 1996; Sadovsky et al., 1995)

Analysis of development in the KO embryos indicates that cells initially migrate to form the urogenital ridge at E9.5 in a manner indistinguishable from wild type littermates. By E11.5, however, the adrenal and gonadal components of the urogenital ridge have failed to form the separate structures. These results were surprising since the loss of expression of known target genes of SF-1 does not explain the developmental defects that occur in the KO mice.

SF-1 KO mice exhibit markedly altered structure in the ventromedial hypothalamus, and the VMH does not form as a discrete nucleus (Ikeda et al., 1995; Shinoda et al., 1995). Further characterization of the VMH has revealed altered cell distribution of a number of immunoreactive cell populations. Normal exclusion of GABA from the developing VMH is not seen in SF-1 KO mice (Dellovade et al., 2000). Cells that express neuropeptide Y, estrogen receptor α, and galanin have altered cell distribution (Dellovade et al., 2000). In addition islet-1 and nkx2.1 also have altered distribution (Davis et al., 2004).

Since SF-1 expression is restricted to the gonadotropes in the pituitary (Shinoda et al., 1995), the pituitary gland develops normally. In the SF-1 null mice, the gonadotropes are still present but have impaired function, as evidenced by the

absence of LHβ, FSHβ and GnRH-R (Ingraham et al., 1994; Shinoda et al., 1995). The SF-1 KO spleen was smaller than the normal spleen and further examination revealed altered architecture and an immature vascular system (Morohashi et al., 1999). The abnormal developmental features were detected as early as E14.5. However, despite these abnormalities, a role of SF-1 in the spleen has not been defined.

Knock out mice can be rescued by adrenal transplants

SF-1 KO mice can be rescued from postnatal death with adrenals transplanted from wild type littermates. Observation of these adrenal-rescued mice showed that they become obese by three months, mainly due to decreased activity as measured by spontaneous running wheel activity instead of increased food consumption (Majdic et al., 2002). The adrenal rescued mice developed a more severe obesity than ovarectimized mice, suggesting that the disruption of the VMH formation, rather than merely the lack of estrogen, could play a role in the development of obesity.

Heterozygous Phenotypes

Heterozygous SF-1 KO mice exhibit impaired stress response due to delays in adrenal development (Bland et al., 2000). The adrenals in +/- mice are decreased in size compared to +/+ mice and response to stress is impaired as measured by production of corticosteroids in response to food deprivation, immobilization or inflammatory stress (Bland et al., 2000). No SF-1 heterozygote phenotype has been observed in the VMH.

Tissue specific KO of SF-1

Pituitary specific KO reveals essential function of SF-1 in the gonadotropes

Using a transgene in which Cre recombinase was driven by αGSU, a loxP-modified SF-1 locus was selectively inactivated in gonadotropes (Zhao et al., 2001). The mice analyzed employed one recombined allele of SF-1 with one floxed allele of SF-1 (F/R). This strategy produced mice that were sterile and failed to develop secondary sexual characteristics. The testis and ovaries of these mice were severely hypoplastic, but the adrenals, VMH, and pituitary appeared grossly intact. The mice had normal levels of all pituitary hormones with the except of LH and FSH, which were decreased (Zhao et al., 2001). Treatment of the mice with exogenous gonadotropins partially rescued the gonadal abnormalities, establishing that the reproductive phenotype occurs due to lack of SF-1 in the gonadotropes (Zhao et al., 2001). These results are of interest because they establish the essential role of SF-1 in the gonadotropes in the context of intact gonads.

Gonad specific KO reveals the essential role of SF-1 in reproductive development and function

An anti-Müllerian hormone type 2 receptor knock-in cre allele was used to selectively inactivate SF-1 in the Leydig cells in testes and granulosa cells in the ovary (Jeyasuria et al., 2004). To facilitate SF-1 inactivation, one null allele of SF-1 was used in addition to one floxed allele. The adrenal glands of these mice were functional, although smaller as seen previously with SF-1 +/- mice, as the mice survived without exogenous corticosteroids. In adult males, the testes remained at

the level of the bladder and had impaired spermatogenesis. Abnormalities of the testes were seen by as early as E14.5, when the KO testis were small than WT and did not contain discernible testicular cords. The cords later were visible by E16.5. At all stages examined, the testes were smaller in the KO mice than the WT mice (Jeyasuria et al., 2004). The developing ovary was indistinguishable in WT and KO mice. The adult female mice were sterile and their ovaries contained hemorrhagic cysts and lacked corpus lutea. This phenotype is reminiscent of that seen in the aromatase and estrogen receptor α KO mice, suggesting impaired estrogen production. The ovary was correctly positioned in the female and development of the ovaries was not obviously impaired; thus the adult phenotype likely develops in the postnatal period (Jeyasuria et al., 2004). The important results were that the Leydig specific SF-1 KO causes developmental delay *in utero* in males and impaired postnatal gonadal function in both sexes but does not cause complete loss of the gonads. This is important because in the global KO, the agenesis of the gonads prevents further study, while the development of the tissue specific mice allow for the developmental effects of SF-1 to be studied.

Brain specific KO of SF-1 results in metabolic syndrome and increased anxiety

Using a nestin-cre transgene, a CNS-specific KO of SF-1 was made. Although complete characterization of these mice is ongoing, the absence of SF-1 in these mice leads to mislocalization of cells in the VMH (Davis et al., 2004). The mislocalization is less severe than that in the global SF-1 KO, perhaps because the nestin-cre does not inactivate SF-1 until E12-E13. The CNS-KO mice have

decreased locomotor activity, became obese on a high-fat diet, and have a significant “anxious” behavior (L.Zhao, personal communication).

1.4 Transcriptional Regulation of SF-1

Global Regulation of SF-1 Expression

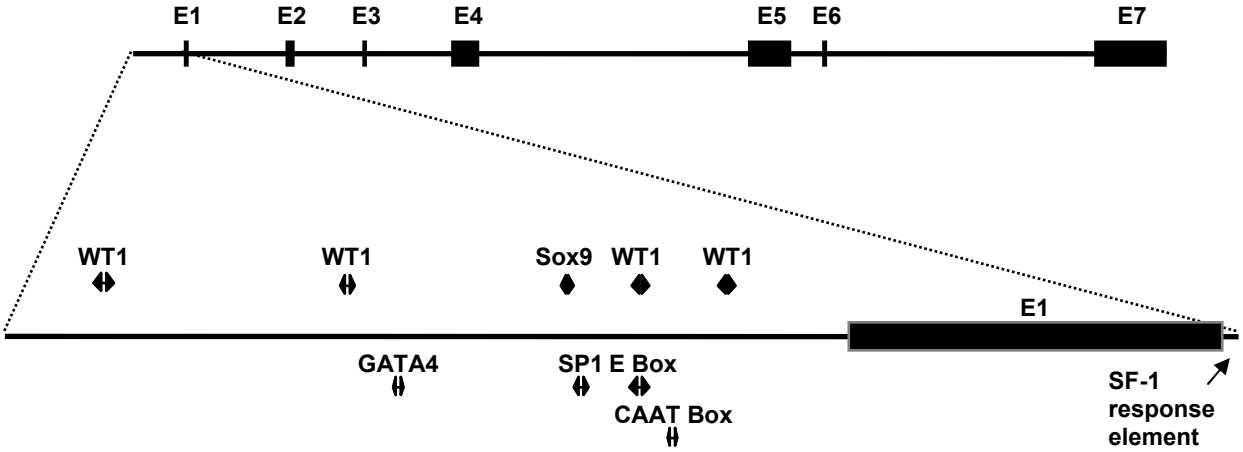


Figure 1-3 Schematic diagram of the *Nr5a1* locus.
Shown is the structural organization of the mouse *Nr5a1* gene.
The expanded region shows elements in the proximal promoter that have been implicated in SF-1 expression.

Regulation of the complex expression of SF-1 is not well understood. Most studies of the SF-1 promoter have utilized transient transfection experiments in cultured cells to define important regulatory elements in the proximal promoter region. A summary of defined transcription factor binding sites is diagrammed in Figure 1-3. Studies have identified several elements that regulate SF-1 expression, including an E box that is essential for SF-1 expression (Harris and Mellon, 1998; Nomura et al., 1995), a binding site for GATA4 (Tremblay and Viger, 2001), a Sox9 site (Shen and Ingraham, 2002), a SF-1-responsive element (Nomura *et al.*, 1996), a CAAT box site that works in synergy with the E box (Woodson et al., 1997), and

three Sp1 sites. As these explorations of the promoter cannot fully account for the expression pattern that is exhibited by SF-1 since many of these proteins are also expressed in cells and tissues in which SF-1 is not expressed.

There are some studies that look at the regulation of SF-1 expression using knockout mouse models. One such model is the Wilms tumor (WT1) KO mice. The WT1 KO mice have gonads that fail to develop beyond E11. In the WT1 KO mice, the expression of SF-1 is absent and is indeed never initiated in the gonads at E9.5 (Wilhelm and Englert, 2002). Another such model is the LIM homeobox gene Lhx-9 KO mice. Mice lacking Lhx9 fail to develop gonads although development is normal until E12.0 (Birk et al., 2000). Expression of SF-1 in these mice is reduced in the gonads at E11.5, but is unchanged in the adrenal precursor population (Birk et al., 2000). One gene that is suggested to repress the transcription of SF-1 is basic helix loop helix protein, Pod1. Pod1 knockout mice, in addition to kidney and other defects, do not properly develop gonads and the external genitalia is female irrespective of genetic sex (Cui et al., 2004). SF-1 and Pod1 are normally expressed in distinct populations within the urogenital ridge. In Pod1 KO mice, the expression of SF-1 expands beyond its normal expression boundries to the mesonephros (Cui et al., 2004). Pod1 is able to repress the promoter of SF-1 in a dose dependent manner (Cui et al., 2004; Tamura et al., 2001). These results suggest that the expression of Pod1 represses the expression of SF-1.

SF-1 autoregulation of gene expression

The first intron of SF-1 contains an element to which SF-1 can bind, as evidenced by EMSA and DNase footprinting (Nomura et al., 1996). Mutation of this

site in transient transfections decreases reporter gene activity (Nomura et al., 1996). In addition, DNase I hypersensitivity mapping reveals that the chromatin surrounding this site is open (Ninomiya et al., 1996; Nomura et al., 1996). However other reports suggest that the potential SF-1 binding site in the first intron does not enhance transcription (Harris and Mellon, 1998; Oba et al., 2000).

The Nr5a1 locus contains two hypersensitive sites

Several potential regulatory regions have been mapped by sensitivity to DNase I. One site is in the first intron of SF-1 and contains the SF-1 binding site mentioned above (Ninomiya et al., 1996; Nomura et al., 1996). This site is open in Y1 and adrenal cells and closed in liver, ECA2, and 3T3 cells. The second region is 3' to exon 7 of SF-1 (Ninomiya et al., 1996). This site is open in Y1 and ECA2 cells and closed in 3T3 cells. DNaseI footprinting identified a protected region that is similar to a SF-1 consensus binding site, but no functional data regarding this site have been published.

Tissue and Cell Specific Regulation of SF-1 Expression

Alternative promoter usage results in pituitary specific transcript

ELP3, which differs in the 5'-UTR from SF-1, is the primary transcript produced in pituitary (Ninomiya et al., 1995). Two alternative promoters for SF-1 were found in rat and may account for the tissue specific distribution of SF-1 expression in spleen and pituitary gonadotropes (Kimura et al., 2000). However only one of these transcripts was expressed in mouse and human, suggesting species specific usage

of the promoters (Kimura et al., 2000). Functional importance of these promoters *in vivo* has yet to be assessed.

1.5 SF-1 Target genes

Position within consensus sequence	1	2	3	4	5	6	7	8	9	10
A	0.12	0.17	0.05	**0.86**	**0.98**	0.00	0.00	0.05	0.07	**0.79**
T	0.17	**0.48**	0.00	0.00	0.00	0.00	0.00	**0.69**	0.17	0.05
G	**0.46**	0.09	0.14	0.14	0.00	**1.00**	**1.00**	0.02	0.02	0.07
C	0.25	0.26	**0.81**	0.00	0.02	0.00	0.00	0.24	**0.74**	0.09
Consensus	**G**	**T**	**C**	**A**	**A**	**G**	**G**	**T**	**C**	**A**

Figure 1-4 SF-1 Binding Site Frequency Matrix
Modified from Busygina et al., 2003

SF-1 binding sites

SF-1 binds DNA as a monomer and the extended consensus binding site of SF-1 is GTCAAGGTCA. In all binding sites identified to date, the guanines are absolutely required, while the remaining nucleotides can vary although the two alanines preceding the guanines have a very high conservation (Busygina et al., 2003). The different binding sites present in the target genes can show differences in response to mutant forms of SF-1 protein, as analysis of human mutations has revealed (discussed below).

Target Genes of SF-1 can be in steroidogenic and non-steroidogenic cells

SF-1 target genes are found in all of the tissues in which SF-1 is expressed. The genes can be separated into those expressed in steroidogenic cells and non-steroidogenic cells. Target genes in steroidogenic cells include cholesterol side-

chain cleavage (CYP11A) (Lala et al., 1992), 17α- hydroxylase (Bakke and Lund, 1995) , aldosterone synthase (Lala et al., 1992), 21-hydroxylase (CYP21) (Lala et al., 1992), steroidogenic acute regulatory protein (StAR) (Caron et al., 1997; Sugawara et al., 1996), dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome (DAX-1) (Burris et al., 1995; Kawabe et al., 1999), 3β-hydroxysteroid dehydrogenase (Leers-Sucheta et al., 1997), Mullerian inhibiting substance receptor (de Santa Barbara et al., 1998), aromatase (Lynch et al., 1993), adrenocorticotropin receptor (Cammas et al., 1997), small heterodimer partner (Lee et al., 1999), scavenger receptor, class B, type 1 (SRB1) (Cao et al., 1997), aldose reductase like protein (Aigueperse et al., 2001), insulin like 3 (Koskimies et al., 2002), HMG CoA reductase (Mascaro et al., 2000), prolactin receptor (Hu et al., 1997), and sex determining region Y (SRY) (de Santa Barbara et al., 2001).

Target genes in non-steroidogenic cells include N-methyl D-aspartate receptor (Pieri et al., 1999), neuronal nitric oxide synthase (Wei et al., 2002), Mullerian inhibiting substance (Hatano et al., 1994), luteinizing hormone β subunit (Halvorson et al., 1996), inhibin α (Ito et al., 2000), follicle stimulating hormone receptor (Levallet et al., 2001), oxytocin (Wehrenberg et al., 1994), follicle stimulating hormone β (Jacobs et al., 2003), and gonadotropin-releasing hormone receptor (Ngan et al., 1999). A detailed list of SF-1 target genes, including characterized binding sites and cell lines/cell types characterized, is provided in appendix 1.

1.6 SF-1 Protein

SF-1 has the protein structure of a typical nuclear hormone receptor

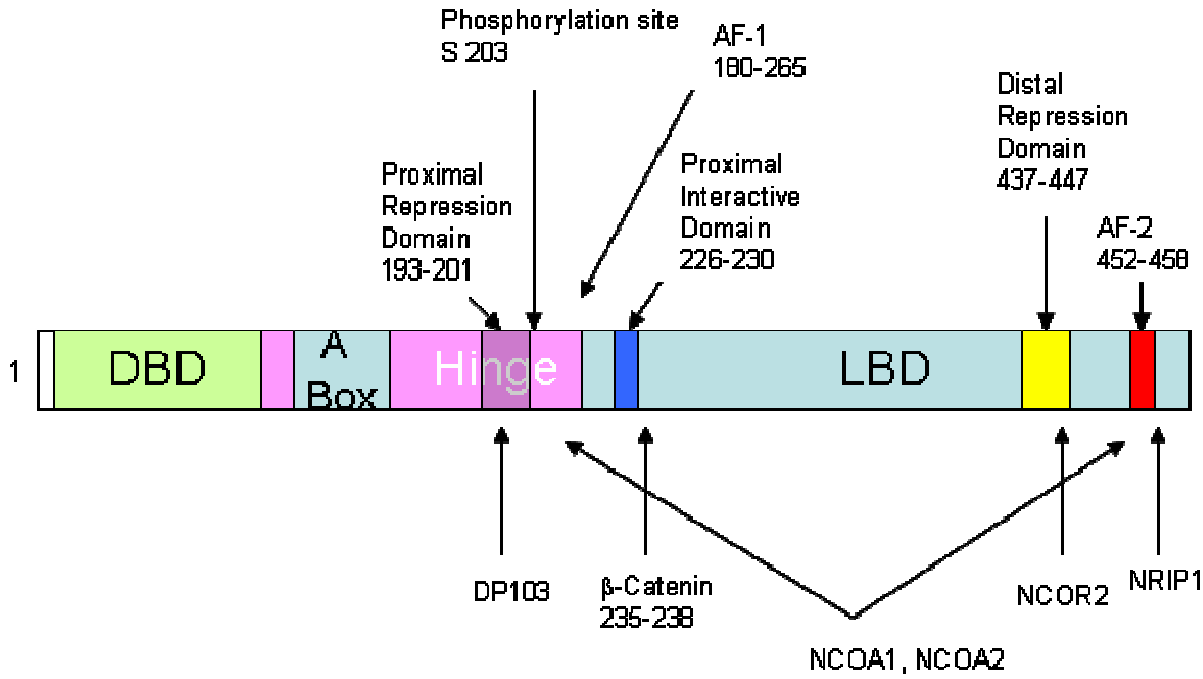


Figure 1-5 SF-1 Protein Domain Organization and Protein/Protein Interface Sites
Diagram of the SF-1 protein. Characterized domains are indicated along with regions of the protein known to interact with other proteins. DBD, DNA binding domain; LBD, ligand binding domain.

SF-1 is an orphan member of the nuclear hormone receptor superfamily (Mangelsdorf et al., 1995). It has a typical nuclear hormone receptor structure with a zinc finger DNA binding domain (DBD) and a ligand binding domain (LBD) (Figure 1-6). The DNA binding domain contains two zinc finger domains that are essential for DNA binding activity. In addition, the nuclear localization signal is also located in this domain and mutations to this domain cause inefficient transport to the nucleus (Hammer et al., 1999). The A box is an accessory DNA binding domain region. A recently characterized human has been found within the A box (Achermann et al., 2002). Although SF-1 is classified as an orphan nuclear receptor, by homology to

other nuclear hormone receptors there is a putative ligand binding domain. The recently solved crystal structures of SF-1 (discussed below) shed a very interesting light on the ligand binding domain and identify a potential ligand for the protein. The SF-1 protein has two activation function (AF) domains, AF-1 and AF-2. The serine residue (S203) at which SF-1 is phosphorylated is located in AF-1 (Hammer et al., 1999). The AF-2 domain contains the hexamer motif (**LL**IEM**L**) found in many nuclear receptors, which is critical for transcriptional activation by SF-1 (Crawford et al., 1997). However, although the hexamer motif is essential for transcription it is not sufficient as another domain, termed the proximal interactive domain which is also required (Crawford et al., 1997). The proximal repression domain is where the interaction between Dax-1 and SF-1 occurs (Ou et al., 2001). This domain is required for the repression of SF-1 by Dax-1 and the recruitment of NCOR2 (nuclear receptor corepressor 2). The distal repression domain also exhibits transcriptional repressor activity through the interaction with DP103 (Crawford et al., 1998).

Phosphorylation and acetylation of SF-1 regulate transcriptional activity

SF-1 is phosphorylated at Ser-203 and mutation of this serine to an alanine reduces the activation potential of SF-1 by 50% (Hammer et al., 1999). This phosphorylation is potentially mediated by a protein in the ras/MAPK pathway (Hammer et al., 1999). Phosphorylation of the AF-1 domain enhances the recruitment of cofactors (Hammer et al., 1999). SF-1 is acetylated *in vivo* by the acetylase GCN5 and this acetylation of the SF-1 protein stimulates its transcriptional activity (Jacob et al., 2001).

Crystal Structures of SF-1 reveal a potential ligand for SF-1

Two papers have recently been published with the solved crystal structure of the ligand binding domain of SF-1. The first paper described a crystal structure of the ligand binding domain with a phosphatidylinositol in the ligand binding pocket of both mouse and human SF-1 (Krylova et al., 2005). The phosphatidylinositol is required for maximal activity of SF-1, as measured in a luciferase reporter gene assay using the mouse aromatase promoter as the target gene (Krylova et al., 2005). The second crystal structure found a different putative ligand in the ligand binding pocket of the crystal structure (Li et al., 2005). Li et al. found that the ligand was phosphatidylethanolamine. As seen with the first paper, modifications to the protein sequence designed to modify the size of the ligand binding pocket significantly reduces the ability of SF-1 to activate transcription (Li et al., 2005). The differences in the crystal structures could be due to the protein purification methods employed by each group or due to modification of the protein sequence made for crystallographical purposes (Krylova et al., 2005; Li et al., 2005). Despite these differences, it is apparent that SF-1 has an endogenous ligand that is able regulate and modify the transcriptional activity of the protein. Much work remains to be done to assess the significance of the identified ligands, both *in vitro* and *in vivo.*

Activation of transcription by SF-1 is modified by co-activators and co-repressors

A growing number of protein/protein interactions have been reported for SF-1. These proteins are able to interact with SF-1 in two different ways. The first is through protein/protein interaction on DNA via adjacent promoter elements, resulting

in synergistic effects on the promoter activity; the second is through SF-1 binding to DNA and the second protein interacting with SF-1 independent of the second protein binding to DNA. Many of these protein/protein interactions occur via the LXXLL motif of SF-1. The LXXLL motif is a common sequence in many nuclear receptors that interact with many of the co-regulatory proteins (Jepsen and Rosenfeld, 2002; Savkur and Burris, 2004). Proteins that have been reported to interact with SF-1 include β-catenin (Mizusaki et al., 2003), nuclear receptor co-activator 1 (Crawford et al., 1997; Ito et al., 1998), nuclear receptor co-activator 2 (Borud et al., 2002; Hammer et al., 1999), CREB binding protein (Monte et al., 1998), transcription regulating factor 1 (Gizard et al., 2002), paired-like homeodomain transcription factor 1 (Tremblay et al., 1998), pituitary specific transcription factor 1 (Tremblay et al., 1998), early growth response protein 1 (Halvorson et al., 1998), nuclear factor Y (Jacobs et al., 2003), estrogen receptor alpha (Drean et al., 1996), Wilms' tumor 1 (Nachtigal et al., 1998), small heterodimer partner (Lee et al., 1999), nuclear receptor co-repressor 2 (Crawford et al., 1998), nuclear receptor interacting protein 1 (Mellgren et al., 2003), CCAAT/enhancer binding protein (Reinhart et al., 1999), dead box polypeptide 20, 103KD (Ou et al., 2001), SRY-box containing gene 9 (de Santa Barbara et al., 1998), and Zip67 (Borud et al., 2003) Some of the better characterized interactions are discussed below.

Co-Activators

β-Catenin

β-Catenin (Catnb) has been proposed to interact with SF-1 in a synergistic manner on the Dax-1 promoter (Mizusaki et al., 2003). This interaction between SF-1 and β-catenin is a protein/protein interaction and was mapped to amino acid residues 235-238 in the mouse protein (Mizusaki et al., 2003). It is suggested that since WNT4 (β-catenin is a key signal transducer in the Wnt pathway) is high in the ovary, that could explain the expression of Dax-1 in the ovary, where Dax-1 expression is high and SF-1 expression is relatively low. These results are supported by the observation that Dax-1 is reduced in Wnt4-/- mice (Mizusaki et al., 2003).

β-Catenin and SF-1 interact synergistically on the inhibin α promoter (Gummow et al., 2003). SF-1 binding to the promoter is required for this synergistic activation and β-catenin is bound to SF-1 as demonstrated through chromatin immunoprecipitation (Gummow et al., 2003). The data suggests that the synergism between SF-1 and β-catenin is independent of TCF/LEF, a common β-catenin binding partner (Gummow et al., 2003).

β-Catenin and SF-1 can also interact synergistically on the mullerian inhibiting substance type II receptor promoter (Hossain and Saunders, 2003). Unlike with the inhibin α promoter, this interaction is dependent upon TCF/LEF (Hossain and Saunders, 2003). Mutation of the SF-1 binding sites in the promoter disrupt the synergism between β-catenin, TCF/LEF and SF-1 (Hossain and Saunders, 2003).

Nuclear Receptor Coactivator 1

Nuclear Receptor Coactivator 1 (NCOA1; steroid receptor coactivator-1, SRC-1; Receptor Interaction Protein 160kDa, RIP160) (Crawford et al., 1997; Ito et al., 1998) enhances the activity of SF-1. This interaction between SF-1 and NCOA1 is dependent upon both the AF2 motif of SF-1 and an additional region of the protein termed the AF1 (Figure 1-5).

CREB Binding Protein and p300

Regulation of the human p450scc gene by SF-1 can be modulated through interaction with CREB binding protein (CBP) and p300 (Monte et al., 1998). P300 can regulate the synergy between Egr-1 and Sf-1 on the LHβ promoter and chromatin immunoprecipitation shows the presence of all three proteins on the LHβ promoter (Mouillet et al., 2004).

Transcriptional Regulating Factor 1

The human p450scc gene regulation by SF-1 and CBP/p300, can be further enhanced through interaction with transcriptional regulation factor 1 (Trerf1; TReP-132) in NCI-H295 cells (Gizard et al., 2002). Mouse Trerf1 is expressed in the VMH in a pattern that is very similar to that of SF-1 (Duguay et al., 2003), although a function of Trerf1 is currently unknown in this expression site.

Nuclear receptor coactivator 2

AF-1 and AF-2 domains are required for SF-1 interactions with nuclear receptor coactivator 2 (Figure 1-5) (NCOA2 ; transcriptional intermediary factor 2, TIF2; glucocorticoid receptor-interacting protein-1, GRIP1) (Hammer et al., 1999).

Phosphorylation of SF-1 greatly increases the interaction with NCOA2. An artificial promoter construct containing 5 binding sites from the 21-hydroxylase gene had activity enhanced by SF-1 and NCOA2 in a dose dependent manner, while a phosphorylation mutant of SF-1 is unable to be enhanced by the presence of NCOA2 (Hammer et al., 1999)

Paired-like homeodomain transcription factor 1

Paired-like homeodomain transcription factor 1 (Pitx1; Pituitary homeobox 1, Ptx1; blackfoot, Bft; pituitary OTX-related factor, P-otx) is expressed in most pituitary derived cells, but is the only Ptx protein in the corticotropes and is predominant in gonadotrophes (Tremblay et al., 1998). Ptx activates most pituitary hormone promoters and appears to be a general regulator of pituitary specific transcription (Tremblay et al., 1998). Strong synergistic effects are observed on the βLH promoter with Ptx1 and SF-1, but not on the βFSH, α GSU or GnRH-R promoters (Tremblay et al., 1998). Synergistic activation between SF-1 and Ptx1 requires SF-1 binding to the DNA, but Ptx1 DNA binding does not need to occur as long as a protein/protein interaction is present (Tremblay et al., 1999).

SRY-box containing gene 9

SRY box containing gene 9 (Sox9) and SF-1 can synergistically activate the MIS promoter (de Santa Barbara et al., 1998). SF-1 and Sox9 proteins are able to interact in the absence of DNA and this interaction occurs through the SF-1 c-terminal and the Sox9 DNA binding domains (de Santa Barbara et al., 1998).

Synergistic activation of the MIS promoter requires binding of SF-1 to the MIS promoter.

Early Growth Response 1

Early growth response 1 (Egr-1; tetradecanoyl phorbol acetate-inducible sequence 8, TIS8; G0/G1 switch gene 30, G0S30; nerve growth factor-induced A, NGFI-A; ZNF225; KROX-24; Zinc finger protein 225, ZNF225) and SF-1 interact synergistically on the LHβ promoter (Halvorson et al., 1998). Through GST pulldown experiments it was demonstrated that SF-1 and Egr-1 have direct protein/protein interactions. There are two sets of paired SF-1 and Egr-1 binding sites on the LHβ promoter and mutation of some of the sites will blunt the synergistic activation of the promoter by SF-1 and Egr-1, showing that this synergy is greatest when both proteins are bound to DNA and interacting with each other (Halvorson et al., 1998).

Nuclear factor Y

The mouse β-FSH promoter has a nuclear factor Y (NF-Y) and SF-1 binding sites and mutation of all of these sites reduces basal activity in an additive manner more than each single mutation (Jacobs et al., 2003). NF-Y subunit A and SF-1 can interact physically in a GST pull down assay, showing direct protein/protein interaction in addition to binding of the proteins to DNA (Jacobs et al., 2003).

Wilms' Tumor 1

Four major isoforms of Wilms' tumor 1 (WT1) are generated by alternative spicing and all of the isoforms are expressed in WT1 expressing tissues. One isoform, termed –KTS, is able synergize with SF-1 on the MIS promoter (Nachtigal

et al., 1998). This synergy was due to protein/protein interaction and was dependent upon SF-1 binding to DNA, but WT was not bound to DNA (Nachtigal et al., 1998). Mutations of WT1 commonly found in Denys-Drash syndrome, commonly characterized by male pseudohermaphroditism and urogenital defects, were unable to synergize with SF-1 (Nachtigal et al., 1998). Dax-1 (discussed below) was able to disrupt to synergism between WT1 and SF-1 although Dax-1 and WT1 interact with SF-1 through different regions of the protein (Nachtigal et al., 1998).

Co-repressors

Dosage-Sensitive Sex Reversal-Adrenal Hypoplasia Congenita Critical Region on the X chromosome, Gene1

Dax-1 (Dosage-Sensitive Sex Reversal-Adrenal Hypoplasia Congenita Critical Region on the X chromosome, Gene1) and SF-1 expression are mostly co-localized through embryonic and postnatal development (Ikeda et al., 1996; Ikeda et al., 2001; Kawabe et al., 1999). Dax-1 is expressed in the adrenal cortex, Leydig cells, theca and granulosa cells, anterior pituitary and the VMH of adult mice (Ikeda et al., 1996). Dax-1 is expressed in the urogenital ridge, throughout gonadal and adrenal development in a pattern very similar to that of SF-1 (Ikeda et al., 1996). The Dax-1 promoter contains SF-1 binding sites and Dax-1 expression is impaired in SF-1 KO mice (Ikeda et al., 1996; Kawabe et al., 1999) suggesting the Dax-1 is a target gene of SF-1. Dax-1 is mutated in human cases of congenital adrenal hypoplasia and hypogonadotropic hypogonadism and duplication of the gene causes dosage sensitive sex reversal. There is a wide phenotypic spectrum exhibited by

these mutations, and like human SF-1 mutations (discussed below), much is still to be learned about the role of Dax-1 in human development.

As a target gene of SF-1 it is interesting that Dax-1 can function as a repressor of SF-1 mediated transcription. Dax-1 has been shown to repress SF-1 mediated transcription of the Insl-3 (Koskimies et al., 2002), Cyp17 (Hanley et al., 2001), Cyp19 (aromatase) (Wang et al., 2001), and LHβ (Dorn et al., 1999) genes. The repression of SF-1 promoter activation by Dax-1 is most likely a feature that can occur on all SF-1 target genes.

Dax-1 interacts with SF-1 through LXXLL -related motifs in Dax-1 (Suzuki et al., 2003). The Dax-1 LXXLL motifs and SF-1 play an important role in the subcellular localization of Dax-1. In the absence of SF-1, Dax-1 has a diffuse distribution pattern. In the presence of SF-1, Dax-1 has a nuclear distribution that depends on the LXXLL motifs (Kawajiri et al., 2003).

Nuclear Receptor Co-repressor 2

NCOR2 (nuclear receptor corepressor 2; N-CoR; Silencing Mediator for Retinoid and Thyroid hormone receptors, SMRT) is a corepressor molecule that interacts with many nuclear receptors. Dax-1 serves as an essential adapter molecule between SF-1 and NCOR2, but congenital adrenal hypoplasia (ACH) mutations of Dax-1 cannot recruit NCOR2 (Crawford et al., 1998). This interaction adds one more variable in the regulation of signaling by SF-1 and repression by Dax-1.

DEAD (Asp-Glu-Ala-Asp) box polypeptide 20

DP103 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 20 (DDX20, DP103; Component of gems 3, GEMIN3; Regulator of Steroidogenic Factor-1, ROSF-1) can directly interact with SF-1 through a proximal repression domain and mutations in this domain abrogate this interaction (Ou et al., 2001). DP103 is expressed in the testis, and in lower levels in the adrenal, kidney, brain, placenta, ovary as well as several model cell lines such as Y1, MA-10, and LβT2 (Ou et al., 2001). SF-1 mediated activation of a rat P450scc promoter construct with two SF-1 binding sites at -79 (SCC2; GGGAGGTCA) and -51 (SCC1; TCAAGGCTA) was repressed in a dose dependent manner by the addition of DP103 (Yan et al., 2003). Similar results were seen with P450scc and P450c21 when measured at the RNA level in Y1 cells transfected with a DP103 plasmid, however RNA levels of StAR were not repressed by transfection of DP103 (Yan et al., 2003). Levels of SF-1 also remained unchanged with transfection of the DP103 plasmid. These results suggest that DP103 is able to repress SF-1 mediated transcription in a promoter independent manner.

Nuclear Receptor Interacting Protein 1

Nuclear receptor-interacting protein140 (NRIP1; receptor interacting protein, RIP140) interacts with the SF-1 AF-1 domain through LXXLL motifs (Mellgren et al., 2003). NRIP1 was shown to interact with both SF-1 and Dax-1 and inhibit the activity of the human StAR promoter in the presence of SF-1 (Sugawara et al., 2001). A different study showed that the ability of NRIP1 to suppress SF-1 mediated transcription was dependent on the cell line and promoter construct used for the

experiments. Using a bovine -290Cyp17 reporter construct with one SF-1 binding site (TGAGCATTAACATAAAGTCAAGGAGAAGGTCAGGG) and a 1.3kb human StAR promoter it was shown that NRIP1 overexpression could repress Cyp17 and StAR activity in Y1 cells (Mellgren et al., 2003). Experiments were also performed in COS-1 cells overexpressing both NRIP1 and SF-1 and cotransfected with promoter constructs for bCyp17, bCyp11A, bCyp11B, mMis and hStAR. NRIP1 was able to reduce the SF-1 stimulated reporter activity on all promoters except the bCyp11A (Mellgren et al., 2003). It is possible to partially inhibit the NRIP1 repression of SF-1 through the use of TSA, which inhibits HDACs (Mellgren et al., 2003). This is another example of a co-repressor that functions in a promoter specific manner.

Zinc finger protein 67kDa

Zip67 (Zinc finger protein 67kDa), was cloned using a yeast two hybrid system where the bait was the C terminus of SF-1 (Borud et al., 2003). Transfections demonstrated that Zip67 could repress the SF-1 mediated transcription from SF-1 target promoters (Borud et al., 2003). This interaction was dependent on the AF2 domain of SF-1 (Borud et al., 2003).

1.7 Liver receptor homolog 1

Liver receptor homolog 1 (LRH-1; Nr5a2; fetoprotein transcription factor, Ftf) is the most closely related member of the nuclear hormone receptor superfamily to SF-1. LRH-1 has a similar domain structure to SF-1, with a DNA binding domain (DBD), an A box, ligand binding domain (LBD), and AF-2 motif (Fayard et al., 2004).

Mouse SF-1 and LRH-1 share 76% identity in their LBDs and 95% identity in the DBD.

LRH-1 binds to the same basic consensus sequence of PyCAAGGPyCPu as SF-1 and activates transcription of several of the same genes. Some of the target genes of LRH-1 include cholesterol 7-α-hydroxylase (Lu et al., 2000), aromatase (Clyne et al., 2002), hepatitis B virus enhancer (Li et al., 1998), hepatocyte nuclear factor(HNF) 1α (Pare et al., 2001), HNF3β (Pare et al., 2001), HNF4α (Pare et al., 2001), adiponectin (Iwaki et al., 2003), steroidogenic acute regulatory protein (StAR) (Kim et al., 2004), apolipoprotein A1 (Delerive et al., 2004), ABCG5/ABCG8 (Freeman et al., 2004), 3β-hydroxysteroid dehydrogenase type II (HSD3B2) (Peng et al., 2003), and scavenger receptor class B type I (SR-BI) (Schoonjans et al., 2002).

LRH-1 is expressed in the liver (Galarneau et al., 1996) , pancreas (Annicotte et al., 2003), ovary (Falender et al., 2003; Hinshelwood et al., 2003), adipocyte (Clyne et al., 2002), placenta (Sirianni et al., 2002), adrenal (Wang et al., 2001), and testis (Pezzi et al., 2004). LRH-1 KO embryos die at E6.5-7.5 most likely from endoderm disruption (Pare et al., 2004).

Much like SF-1, LRH-1 interacts with a growing list of proteins that act as both co-activators and co-repressors. Some of these interacting proteins are β-catenin (Botrugno et al., 2004), Dax-1 (Suzuki et al., 2003), prospero related homeobox (Prox1) (Qin et al., 2004), small heterodimer partner (SHP) (Lee and Moore, 2002), nuclear receptor co-activator 1 (Xu et al., 2004), and nuclear co-repressor 2 (Xu et al., 2003). Not surprisingly, most of these proteins also have characterized interactions with SF-1.

1.8 Human Mutations of SF-1

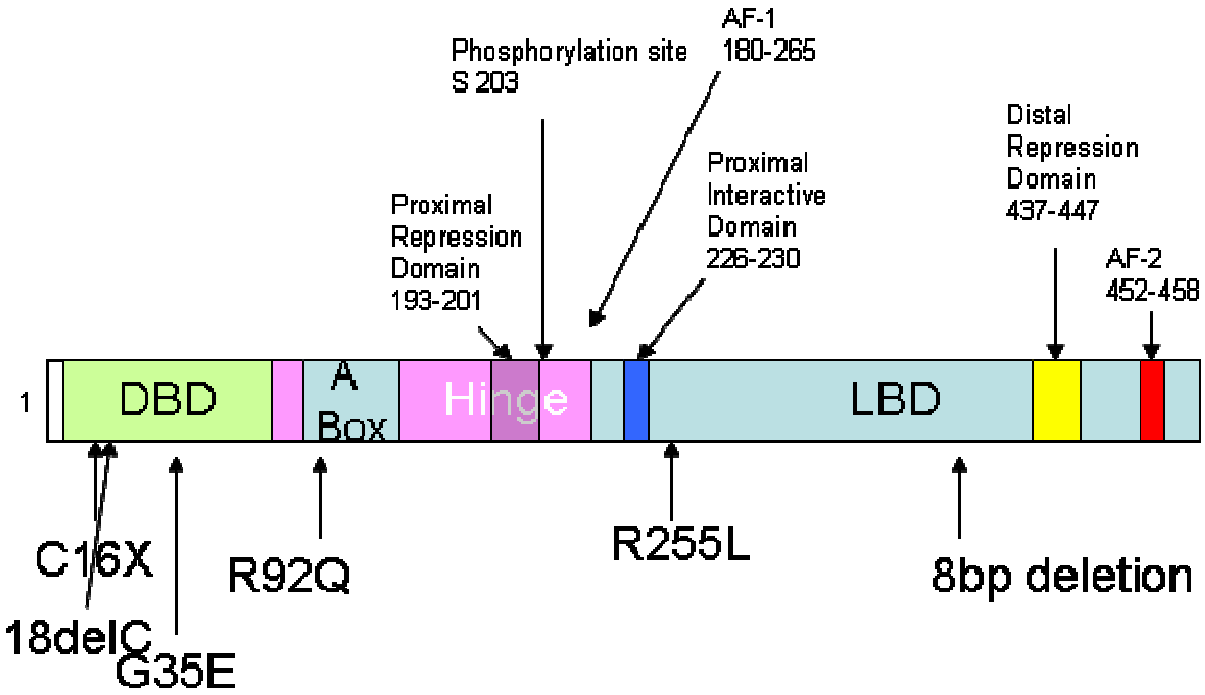


Figure 1-6 Location Human Mutations in the SF-1 Protein
Known human mutations are indicated on the SF-1 protein. Mutations are their effects are discussed in the text

G35E

The first human SF-1 mutation to be described was a heterozygous mutation at G35E (Achermann et al., 1999). The patient had complete XY sex reversal with adrenal insufficiency. Normal Mullerian structures (e.g. oviduct and a uterus) were detected, as were streak gonads (Achermann et al., 1999). This patient became obese in late adolescence (Ozisik et al., 2002). In vitro or in cultured cells, the mutation does not interfere with protein stability, translation or nuclear localization, but it eliminates binding to the Cyp11a promoter (GGAGGTCA and CAAGGCTA) (Achermann et al., 1999). Further examination revealed that the mutant protein

also could not bind to rat LHβ (ACAAGGTCA and GCAAGGCCA) or mouse DAX-1 composite sequence (TCGAGGTCATGGCCA) or activate promoters containing these sequences (Ito et al., 2000). On the other hand the G35E mutant bound the mouse MIS (CCAAGGTCA) and rat aromatase (CCAAGGTCA) promoter and activated promoter constructs with this sequence with 50% the efficiency of the wild type protein (Ito et al., 2000).

The SF-1 G35E mutant does not synergize with GATA4 on the MIS promoter, despite a retention of the protein-protein interaction between SF-1 and GATA4 (Tremblay and Viger, 2003). The G35E mutation may act as a dominant-negative competitor, since it disrupts synergism between the wild type SF-1 and GATA4 (Tremblay and Viger, 2003) .

R255L

The patient was diagnosed with adrenal insufficiency at 14 months. She had a 46,XX karyotype with normal uterus and ovaries (Biason-Lauber and Schoenle, 2000). Her LH and FSH levels were normal and there was no evidence of abnormalities of the reproductive system. A heterozygous mutation was found in exon 4 and that led to a missense mutation (R255L) in the hinge region. The mutation does not affect protein translation or stability, but the mutant protein does not bind to the SF-1 response element in human CYP11A promoter or activate a reporter construct with these binding sites (Biason-Lauber and Schoenle, 2000). The mutation is not in the zinc finger or A box, so it is unclear why this protein does not bind DNA.

R92Q

The patient has a 46,XY karyotype and is phenotypically female. She presented with primary adrenal failure (Achermann et al., 2002), with a hypoplastic left adrenal and agenesis of the right adrenal. Unlike the other mutations of SF-1, this is a homozygous mutation, as heterozygous parents and sister appear to be normal. The R92Q mutation is in the A-box accessory DNA binding region, and is a conserved residue in other species. The R92Q mutant has impaired binding and transactivation of the Cyp11a promoter (Achermann et al., 2002). The loss of function is not as severe as the G35E mutant on the Dax-1, Cyp19, or Cyp11a promoters (Achermann et al., 2002)

Exon 6 microdeletion

This 46,XY patient presented with gonadal agenesis but normal adrenal function (Correa et al., 2004). Analysis of SF-1 revealed a heterozygous 8 bp deletion (∆8SF-1) that results in a premature stop codon upstream of the AF2 domain (Correa et al., 2004). DNA binding of the ∆8SF-1 is reduced to below that of WT SF-1 on the -140 Cyp21 element (CAAGGCTG) (Correa et al., 2004). The mutation does not change the stability or nuclear location of SF-1. In MA-10 cells, the mutant protein was unable to activate transcription on the Cyp17 promoter, and inhibited transcriptional activation by the WT SF-1 in a dose dependent manner. In H295R cells, ∆8SF-1 augmented the transcriptional ability of WT protein in a dose dependent manner. It is possible that these differences in the cell lines reflect differences in coactivators and corepressors.

C16X

The C16X mutation is a de novo heterozygous mutation causing gonadal dysgenesis with normal adrenal function (Mallet et al., 2004). This mutation causes a termination of translation that should result in a markedly truncated protein. The patient presented at birth with ambiguous genitalia, hypospadias and micropenis. At the age of 6 years and 9 months, adrenal function was re-examined and no abnormalities were seen. The phenotype of the patient is likely the result of haploinsufficiency.

18delC

The 18delC mutation was a heterozygous mutation resulting in a frameshift mutation at the sixth codon and a termination codon at the 74th codon (Hasegawa et al., 2004). The patient was 46, XY with gonadal dysgenesis and normal adrenal function. The patient was moderately obese. The mutation is such that any functional domains of SF-1 would not be contained in any resultant protein. Transfections using the mutant form of SF-1 failed to produce any immunoreactive protein. Reporter assays employing the human Cyp11a promoter and the mutant protein showed that the mutant protein was unable to activate transcription of this SF-1 target gene and did not have any dominant negative activity (Hasegawa et al., 2004). These results suggest that the observed phenotype in the 18delC patient is due to haploinsufficiency of SF-1.

The human mutations lend considerable insight into the importance of the dose-dependent action of SF-1. Five of the six mutations characterized to data are heterozygous mutations, suggesting that human gonadal development is more

sensitive to the dosage of SF-1 than that of mice, as heterozygous mice are fertile. An important difference between the KO mice and the human mutations is the complete lack of SF-1 protein in the KO mice, a condition that has not yet been described in human. Also several of the human mutations result in a presumed dominant negative effect of the protein, a condition that has not been created or characterized in mice. One intriguing fact revealed from the human mutations, is the disruption of binding to some DNA elements, while retaining the ability to bind to other sites. Despite these differences, the human mutations are providing important clues into the function of SF-1 and the important role of gene dosage in proper development.

1.9 Rationale behind the present work

Creation and characterization of eGFP transgenic Mice

As a tool for identifying SF-1 expressing cells in both WT and KO mice, a transgene was created and characterized that expresses enhanced green fluorescent protein (eGFP) using 50 kb 5' of the SF-1 promoter and translation initiation site. A marker of SF-1 expressing cells will be an extremely useful tool for studying the fate of cells in SF-1 KO mice. Expression of the transgene was characterized and pilot experiments were performed to evaluate the usefulness of the transgene to isolate cells by FACS. The transgene will facilitate the identification of novel target genes of SF-1 and analysis of cell migration studies of SF-1 KO cells.

Identification of novel target genes of SF-1

Using the SF-1/eGFP transgene as a marker of cells capable of expressing SF-1, FACS can be used to separate eGFP positive cells from eGFP negative cells. RNA from these cells can be used in Affymetrix Array analysis to profile gene expression. FACS can then be further applied to compare wild type and SF-1 KO cell populations. This will allow us to identify genes with decreased mRNA expression in the SF-1 null mice that are potential target genes of SF-1. Genes identified in this manner can be confirmed with quantitative RT-PCR, *in situ* hybridization, and promoter analysis.

The ventromedial hypothalamic nucleus is an obvious choice for the application of the cell sorting/ novel target gene identification strategy, as there is a paucity of known target genes of SF-1 in the brain. The VMH does not develop in the SF-1 KO mice due to perturbed migration (Davis et al., 2004; Dellovade et al., 2000), but no target genes have been identified that can account for this defect in cell migration. Thus, genes involved in the proper cell migration of the VMH neurons and SF-1 target genes may be identified using this technique.

Analysis of Brain-Derived Neurotrophic Factor as a target gene of SF-1

BDNF was identified as a potential target gene of SF-1 and further characterized. The promoters for brain-derived neurotrophic factor (BDNF) were analyzed for potential SF-1 binding sites. Promoter 4 was identified as having SF-1 binding sites in the proximal promoter. Identified binding sites in promoter 4 were characterized by EMSA and reporter gene assays.

Characterization of a novel SF-1 regulatory element

SF-1 is expressed in a tissue and cell specific manner. However, factors controlling this tissue and cell specific expression have yet to be identified. DNase I hypersensitivity mapping was employed to search for regions of open chromatin in the SF-1 genomic locus. Open regions of chromatin often contain regulatory regions. One such tissue specific hypersensitive site was identified. Genomic alignment with other species showed that the identified mouse sequence had high homology to intronic regions in human, rat, and chicken. EMSA and luciferase assays were employed to characterize this region of genomic DNA

Chapter 2 Methods

2.1 DNA and construct preparation

eGFP Transgene Construction

The SF-1/eGFP transgene contained a 50 kb *Bsi*WI-*Bsi*WI fragment of the SF-1 BAC (Research Genetics, St. Louis, MO) that included ~45 kb of 5'-flanking region from the mouse *Nr5a1* gene, the non-coding first exon, the first intron, and 32 nucleotides from the second exon. As a result of manipulations involved in the cloning strategy, the transgene encodes a protein containing the first five amino acids from SF-1 (Met-Asp-Tyr-Ser-Tyr) and an Ala fused in frame to the initiator Met from eGFP (CLONTECH Laboratories, Inc., CA). The same residues found in the SF-1 coding sequence are also contained within ELP, such that this transgene potentially could also direct eGFP expression to sites where ELP is expressed. The 3'-splice/polyadenylation signals from bovine growth hormone were amplified by PCR and then inserted 3' of the eGFP coding sequence. The proper orientation of the SF-1/eGFP transgene was verified by DNA sequence analysis, and its ability to encode functional eGFP was verified by fluorescence microscopy of stably transfected Y1 mouse adrenocortical tumor cells (N. Hanley, unpublished observation). The transgene was excised from the pBeLoBAC11 vector by digestion with *Pme* I and resolved by preparative pulsed field gel electrophoresis (PFGE) in a 1% Seaplaque agarose gel. Successful resolution of the transgene fragment from the vector was confirmed by PFGE

analysis. SF-1/eGFP transgenic mice were produced by the National Institute of Child Health and Human Development Core Transgenic Facility at the University of Alabama, Birmingham.

Creation of BDNF promoter constructs

Plasmids containing mouse genomic DNA for BDNF were generously provided by Dr. Luis Parada (UT Southwestern Medical Center). The genomic sequence (Accession NW_000178) was examined for SF-1 binding sites using the MatInspector program (Genomatix; www.genomatix.de). Potential SF-1 binding sites were compared to their positions relative to the various exons of BDNF. Luciferase reporter constructs were designed with the potential SF-1 binding sites in mind. The promoter 4 construct contained a SmaI/EcoRI 746bp fragment blunt ended and ligated to the SmaI site of pGL3. The correct orientation of the promoter fragment was confirmed by sequencing. The SF-1 binding sites in Promoter 4 were mutated using the Quikchange kit (Stratagene) according the manufacturer's recommended protocol. .The primers used to mutate the three potential SF-1 binding sites in BDNF Promoter 4 are listed in Table 2-1.

Table 2-1 Primers used for the Quikchange Mutageneis of BDNF Promoter 4

BDNF-229	5'GAGAGAAGCCAGTGCAA**TT**CGATCAGGGATACC3'
BDNF-144	5'CTGAGCCCCCGCAA**TT**AAAAGGCGCGTCGTC3'
BDNF-651	5'GCACACTCCGGAGA**TT**TCAGGGCGCAAACTCTGG3'

Base pairs that were mutated are in Bold.

Creation of reporter plasmid constructs for DNase I hypersensitive site characterization

A 858 bp *Xba*I-*Eco*RI fragment that contains ~700 bp of 5'-flanking region and a portion of the non-coding first exon was excised from Bac RP238i2 (Invitrogen), treated with the Klenow fragment of DNA polymerase to create blunt ends, and cloned upstream of the luciferase reporter gene into a blunted *Hind*III site in the polylinker of pGL3basic (Promega). Indicated portions of the conserved region were then cloned 5' of this promoter into the *Xho*I site using *Sal*I sites introduced by the PCR primers. Primer sequences are listed in Table 2-2. 1:452 (Forward1 and Reverse 1), 1:72 (Forward 1 and Reverse 4), 51:119 (Forward 4 and Reverse 2), 97:188 (Forward 2 and Reverse 5), 169:245 (Forward 5 and Reverse 3), 225:359 (Forward 3 and Reverse 6), and 341:452 (Forward 6 and Reverse 1). Potential binding sites for known transcription factors were mutated using the Quikchange kit for site directed mutagenesis (Strategene) according to the manufacturer's recommendations. Primer sequences used for mutagenesis are provided in Table 2-2.

Table 2-2 Primers used for creating hypersensitive site reporter constructs

Primer Name	Sequence	Purpose
Forward 1	5'GCGTCGACCAGCTCAGAGGCAGGTAAG3'	PCR cloning
Forward 2	5'GCGTCGACCCGAGGACTTAATCGAAGCTTAA3'	PCR cloning
Forward 3	5'GCGTCGACTGAATAGCAGAGCCACTCGAG3'	PCR cloning
Forward 4	5'GCGTCGACGGCAATCGCAGTGTTTATGAAG3'	PCR cloning
Forward 5	5'GCGTCGACGCATTCCCTTAAGCGTTTGC3'	PCR cloning
Forward 6	5'GCGTCGACTTAAACCGTGAAAAGGGGG3'	PCR cloning
Reverse 1	5'GCGTCGACACATCCCCGGAGGCAGAAGG3'	PCR cloning
Reverse 2	5'GCGTCGACTTAAGCTTCGATTAAGTCCTCGG3'	PCR cloning
Reverse 3	5'GCGTCGACCTCGAGTGGCTCTGCTATTCA3'	PCR cloning
Reverse 4	5'GCGTCGACCTTCATAAACACTGCGATTGCC3'	PCR cloning
Reverse 5	5'GCGTCGACGCAAACGCTTAAGGGAATGC3'	PCR cloning
Reverse 6	5'GCGTCGACCCCCCTTTTCACGGTTTAA3'	PCR cloning
Pbx/Meis Quikchange	5'GTCATTAGTGCCTCTCTCCT**cccgggg**CGCAGTGTTTATGA	Quikchange mutagenes

Primers used to create luciferase reporter constructs as detailed. Red indicates a Sal I site that was used for cloning. Lowercase letter in the Quikchange primer indicates basepairs that were mutated.

Genomic sequence alignment of human, mouse, rat, and chicken sequences using VISTA

Genomic alignments were performed using the VISTA genomic alignment program (Frazer et al., 2004), which aligns genomic sequences from multiple species and allows for easy visualization of conserved regions of DNA. Mouse genomic sequence from the *Nr5a1* locus (Base pairs 16231486-16273359 of NT_039206) was used as the base sequence and human, rat, and chicken genomic sequences were compared to the mouse sequence. The default parameters were used in the sequence alignment.

Local Sequence alignment for identified hypersensitive site

The mouse, human, and chicken sequences were aligned using Clustal W(1.4) from the MacVector program (v6.5.3; Oxford Molecular Group) with the following parameters: Open Gap Penalty=10.0, Extend Gap Penalty=5.0, Delay Divergent=40% and Transitions:Weighted.

Prediction of transcription factor binding sites in hypersensitive site

Transcription factor binding sites were predicted using the Matinspector professional release 6.2.2 and the matrix family library version 3.3, August 2003, with the following parameters: all vertebrates, core=0.75, and matrix similarity=Optimized (Genomatix; www.genomatix.de).

2.2 Generation and maintenance of transgenic and knockout mice

Mice were housed at UT Southwestern Medical Center at Dallas under the guidelines of the Institutional Animal Care and Use Committee.

Genotyping Mice for Transgene, SF-1 KO allele, and SRY

The SF-1 KO mice were generated as previously described (Luo et al., 1994). Mice and embryos were genotyped by PCR for the modified SF-1 allele, genetic sex using the Sry gene, and the presence of the eGFP/SF-1 transgene. Primer sequences are listed in Table 2-1.

To obtain DNA to perform the PCR reaction, a toe or small tail snip was cut from the mouse or embryo and a quick lysis was performed (Conner, 2002). Briefly, 100 µl of 0.05 M NaOH was added to each sample and then incubated at 96°C for 20 minutes. Samples were then cooled to room temperature and vortexed vigorously. 100 µl of 0.05 M NaOH and 40 µl of 1 M Tris, p.H. 8.0 were added and samples were again vortexed. SRY and SF-1 genotypes were performed in the same PCR reaction. The SRY/SF-1 PCR reaction contained in a 20 µl reaction: 1.3 M betaine (Sigma), 25 mM KCl, 250 nM of the SRY forward, SRY reverse, SF-1 forward and SF-1 reverse primers, 350 nM SF-1 neo primer, 200 nM dNTPs, 1X PFU turbo PCR buffer (Stratagene), 1U Jumpstart Taq (Sigma), and 1.15 µl DNA sample. For the eGFP transgene, the following PCR reaction was performed in a 20 µl reaction: 25 mM KCl, 250 nM each primer, 200

nM dNTPs, 1X PFU turbo PCR buffer (Stratagene), 0.5 U Jumpstart Taq (Sigma), and 0.55 µl DNA sample. Primer sequences are provided in Table 2-3.

Table 2-3 Primer sequences used for genotyping mice

eGFP reverse	5'GAATGACACCTACTCAGACAATGC3'
eGFP forward	5'CACCATCTTCTTCAAGGACGAC3'
SF-1 forward	5'ACAAGCATTACACGTGCACC3'
SF-1 reverse	5'TGACTAGCAACCACCTTGCC3'
SF-1 Neo	5'AGGTGAGATGACAGGAGATC3'
SRY forward	5'AGGCGCCCCATGAATGCATT3'
SRY reverse	5'TCCGATGAGGCTGATATTTATA3'G

Timed Matings

Transgenic male and female mice were paired in cages at 6 PM, and noon of the morning on which the copulatory plug was detected was designated day 0.5 of gestation (E0.5). After the mothers were anesthetized, the embryos were harvested by Caesarian section. In all cases, the ages of the embryos were confirmed according to the external criteria described by Kaufman (Kaufman, 1992), and the sex and genotype of embryos were determined by PCR assays as described above.

2.3 Immunohistochemistry and in situ hybridization

Characterization of eGFP and SF-1 expression

Embryos were harvested from the mother and fixed en block in 4% paraformaldehyde (PFA), whereas postnatal mice were anesthetized and then perfused with 4% paraformaldyhyde. Embryos or tissues were embedded in Tissue Tek (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and stored at -80°C until sections were cut on a cryostat (20 µm for brain and 15 µm for other tissues). Expression of eGFP was visualized using a Nikon Optiphot microscope

equipped with a UV light source and filters for fluorescein visualization. The same sections were then analyzed for tissue histology by drying overnight at 37°C, followed by staining/counterstaining with hematoxylin/eosin.

Immunohistochemical analyses were performed as follows: mice were anesthetized and then perfused with 4% paraformaldyhyde, postfixed for 12-16 hours, transferred to 70% ethanol, and then embedded in paraffin using standard procedures. Sections (7 μm) were cut on a microtome and dried overnight at 37°C. An antiserum specific for eGFP was purchased from Novus (Littleton, CO) and used for immunohistochemical detection of eGFP at a 1:2500 dilution according to the supplier's recommendations. The rabbit polyclonal antiserum against bovine SF-1 was a generous gift from Dr. Ken Morohashi and was used as described (Hatano et al., 1994).

In situ hybridization for cerebellin 1 and brain-derived neurotrophic factor

Wild-type and central nervous system-specific SF-1 KO mice were used for the *in situ* hybridization. CNS-specific SF-1 KO mice were used due to their viability post-natally and their availability in the laboratory. Mice with the CNS-specific SF-1 KO were generated using a conditional SF-1 allele (Zhao et al., 2001) and the nestin-Cre transgene (Jackson Laboratories). Mice were anesthetized and perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were post-fixed with 4% PFA in PBS at 4°C overnight, cryoprotected in 30% sucrose for 24 h, embedded in OCT compound, and sectioned at 25 um on a cryostat. *In situ* hybridization was performed on every fourth serial section from three brains each from wild-type and CNS-specific SF-1 KO mice. All

photomicrographic images were captured on a Nikon Optiphot microscope with video capture and were imported into Photoshop.

2.4 Fluorescent activated cell sorting, RNA preparation, array procedures and quantitative real time PCR

Initial experiments to determine viability of FACS using the SF-1/eGFP transgenic mice to sort adrenal and testes cells

Testes were dissected from SF-1/eGFP transgenic mice at 3 and 6 weeks of age and adrenal glands were isolated from adult mice. Single cell suspensions were prepared by collagenase digestion, and eGFP-positive and eGFP-negative cells were resolved by FACS essentially as described (Motoike et al., 2000). Briefly, testes and adrenals were minced into small pieces and then suspended in 1 ml cold PBS. Once all samples were ready for digestion, 1 ml of a prewarmed collagenase (Sigma) solution and 10 µl of 1% DNase(Sigma) was added. Cells were incubated at 37°C for 1 hour with constant agitation at 250 rpm. At each 10 minute interval, cells were dissociated with 10 strokes of a pipet. After one hour, 2 ml of DMEM+10% fetal calf serum were added and thoroughly mixed. This mixture was pelleted and 2 ml of DPBS was added to the cell pellet. Cells were mixed and filtered through a Falcon cell strainer and pelleted. 200 µl of red blood cell lysis buffer (Sigma) was added to the cell pellet and mixed. The cell mixture was layered over 1 ml of FCS and pelleted. The pellet was resuspended in PBS containing 0.5 mM EDTA, 3% fetal calf serum and 5 ng/µl DNAse I. Cells were sorted directly into Trizol Reagent.

RNA was prepared using the Trizol reagent and then was used for semi-quantitative RT-PCR assays as described (Zhao et al., 2001). Previously described conditions and primer sets were used to detect SF-1, the cholesterol side-chain cleavage enzyme, and glyceraldehyde phosphate dehydrogenase (GAPDH), and the post-meiotic germ cell marker Meg-1 was measured as described (Sugihara et al., 1999). Primer sets used for these reactions are listed in Table 2-4.

Table 2-4 Primers used for semi-quantitative RT-PCR

SF-1 Forward	5'AAATTCCTGAACAACCACAGC3'
SF-1 Reverse	5'GCATCTCAATGAGAAGGTTG3'
SCC Forward	5'AGTGGCAGTCGTGGGGACAGT3'
SCC Reverse	5'TAATACTGGTGATAGGCCGCC3'
GAPDH Forward	5'ACCACAGTCCATGCCATCAC3'
GAPDH Reverse	5'TCCACCACCCTGTTGCTGTA3'
Meg-1 Forward	5'AACCTGATGGCTGGCTTGAT3'
Meg-1 Reverse	5'TTTTTCTTTACTTTCCTTGG3'

GFP+ Neuronal Cell isolation by FACS

Timed matings were performed as follows; eGFP transgenic, SF-1 +/- male and SF-1 +/- female mice were paired in cages at 6 PM, and noon of the morning on which the copulatory plug was detected was designated day 0.5 of gestation (E0.5).

Female mice were sacrificed on E16.5 and separate single-cell suspensions were prepared from each individual embryo. After several attempts using the method described above, it became apparent that a different protocol for making a single cell suspension was necessary for embryonic neuronal preparations and the following protocol was developed for these cells. To obtain the VMH, coronal cuts were made behind the optic chiasm at the caudal limit of the medial basal hypothalamus (approximately at the midregion of the median eminence). The slices were maintained in PBS at 4°C until a triangular piece was dissected to

obtain only the hypothalamus. This piece was minced into small fragments and incubated in a 1:1 PBS:trypsin/EDTA mixture with the addition of 10 ng/µl DNAse I (Sigma) with shaking for 30 minutes at 37°C. To inactivate the trypsin, PBS containing 0.5 mM EDTA, 3% fetal calf serum and 5 ng/µl DNAse I was added to the cell suspension, cells were gently pelleted and resuspended in the PBS/FCS/EDTA/DNase I mixture. Cells were filtered using a cell strainer (Falcon) and sorted as described (Stallings et al., 2002). Each embryo was examined for the presence of eGFP-positive cells and eGFP-positive cell counts were recorded for each eGFP-positive embryo. After sorting, the SF-1 and SRY genotypes of eGFP-positive embryos were determined by PCR as described above.

RNA Preparation for FACS or QRT-PCR

Cells were sorted directly into TRIzol (Invitrogen) and total RNA was prepared according to the manufacturer's recommendations. After the final step in the TRIzol protocol, samples were pooled by genotype and purified with RNeasy (Qiagen) columns. Each pool contained equal numbers of FACS cells (10,000-20,000).

Preparation of RNA for Affymetrix array

RNA was prepared for the array using a two-cycle amplification method (Affymetrix Genechip eukaryotic small sample target labeling technical note). Briefly, the pooled RNA samples were used to make cDNA using a first strand synthesis protocol (Superscript II, Invitrogen) and then the second strand of

cDNA was made with a special oligo$(dt)_{24}$ primer containing a T7 RNA promoter site added to the 5' poly(T) tract. The Ambion MEGAscript T7 kit was then used to create cRNA in an *in vitro* amplification reaction and all of the cRNA was used to make first and second strand cDNA from the cRNA. In the final step, the ENZO BioArray HighYield RNA transcript labeling kit was used to make biotin-labeled cRNA in an *in vitro* transcription reaction.

Affymetrix mouse U74 Chip and 430 chip

The biotin-labeled RNA was hybridized to the Affymetrix U74A chip by the Array core facility at UT Southwestern Medical Center. Samples were analyzed prior to hybridization using an Agilent bioanalyzer at the UT Southwestern Array core facility. The hybridization to the mouse 430 chip was performed by Norma Anderson:-Dr. Jay Horton's laboratory.

Array data analysis using Gene Traffic

Data were analyzed with Gene traffic V3.2, using robust multi-array average (RMA) to calculate the values. Briefly, the RMA calculation normalizes the arrays to one another using probe level intensities (Irizarry et al., 2003). The RMA value is an average of background-adjusted, normalized, and log-transformed perfect match values. For the U74A array experiments, the baseline was set to the values from three wild type embryo pools. The three pools were 1) single male embryo, 2) male embryo pool, and 3) female embryo pool. The experimental values were also derived from three pools. The experimental pools were 1) single male SF-1 KO embryo, 2) SF-1 KO male pool and 3) SF-1 KO

female pool. For the mouse 430 array, values were derived from one WT pool and one KO pool of mixed sex embryos.

Real time PCR primer design and primer validation

Primers for each gene were designed using Primer Express Software (Perkin-Elmer Life Sciences). Representative accession numbers and primer sequences are provided in Table 2-5. Primers were designed to span an intron whenever possible. The validation reactions (10 µl) contained 50 ng,10 ng, 2 ng, 0.4 ng, 0.08 ng, 0.016 ng, and 0.0032 ng of reverse-transcribed total RNA, 150 nM of each primer, and 5 µl of SYBR Green PCR Master Mix (Applied Biosystems). All PCR reactions were performed in triplicate on an Applied Biosystems 7900HT. Tissues for the validation reactions were chosen based on expression data from the Genomics Institute of the Novartis Research Foundation (GNF) SymAtlas (http://symatlas.gnf.org/SymAtlas) (Su et al., 2002; Su et al., 2004). Primers were validated by analysis of a standard curve comparison to cyclophillin and dissociation curve for each primer pair.

Real Time RT-PCR

Total RNA was prepared from FACS enriched cells using TRIzol (Invitrogen) and then RNeasy columns (Qiagen) as described for the Affymetrix arrays. QRT-PCR pools were created from 10,000-20,000 cells. For experiments, pools were only compared to other pools created from similar numbers of cells. Total RNA was then treated with DNase I (Roche) and reverse transcribed using the Superscript III system (Invitrogen). QRT-PCR was then

performed essentially as described (Repa et al., 2002) with several modifications. In a final volume of 10µl, the real-time PCR contained 0.05-0.10 ng of reverse-transcribed total RNA, 150 nM of each primer, and 5 µl of SYBR Green PCR Master Mix (Applied Biosystems). All PCR reactions were performed in triplicate on an Applied Biosystems 7900HT, and relative mRNA levels were calculated using the comparative C_T method. Quantification was performed using the $\Delta\Delta$Ct method (Applied Biosystems user bulletin #2). Comparisons were made to wild type embryos using cyclophilin as the reference gene. Wild type expression values were set at 100%.

Table 2-5 Primer pairs used for quantitative real time PCR

SYBR	**Forward**	**Reverse**		
Cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT	NM_008907	
Sez6	CCTCGCTGCTGGCGC	CCCGTGATCGGAGCCTC	NM_021286	
Tbr1	CTTGTGGCAAAGCGGACAC	TCCGGATGCATATAGACCCG	NM_009322	
Shox2	ATGTCAACGTAGGTGCTTTAAGGAT	AGGGCGTCACGTTGCAAT	NM_013665	
Dlx1	GCCTCCTTGGGACTCACACA	TCTTGAACTTGGAGCGTTTGTTC	NM_010053	
Foxg1	CAGGAAGCTCTTTTGCTACATGC	CAGTTGGACCGCAGGCTC	NM_008241	
Eps15	CTGAAAAAGTCAGGGCTTCCA	CAGGACACCTTTGCCATCTGT	NM_009996	
Dab2	CCTTGATGATCAAGCTAACAAATTGA	ATGTCCCCAAACAAATCCATCT	NM_023118	
Ten-c	ACCTGATGGGCAGATATGGG	TCCAATGGAACCAGTTAACGC	NM_011607	
Taqman	**Forward**	**Probe**	**Reverse**	
Cbln	ACACAGGAAAGGCAAAGGGA	CCTGCTACGGGAGACAGCGCAGA	GCCTTCTTCGCAGAGCCAT	NM_019626
Ten-M2	AGACAGAATGAGATGGGAAAGAGG	AACAAAATAACCTGCTGCCACCTCTTCTCTG	GTTGCTCCTGCTGAGCCAC	NM_011856

2.5 DNase I Hypersensitivity Assays

The DNase I hypersensitivity assays were performed essentially as described (Ninomiya et al., 1996). Briefly, cells were dissociated from the culture dishes by digestion with trypsin, washed, and replaced in fresh medium. Cells were then treated with increasing amounts of DNase I and genomic DNA was extracted using phenol/chloroform and precipitated using sodium acetate and ethanol. 15 µg of genomic DNA was digested with *Hind*III, and resolved by electrophoresis in a 1% agarose gel. Following transfer to Nytran membranes

using the Turboblotter system (Schleicher & Schuell) and UV cross-linking, the resulting DNA fragments were detected by hybridization with ^{32}P-labeled probes derived from different regions of the *Nr5a1* locus. Probes were created by cloning *HindIII* fragments for BAC RP238i2 (Invitrogen) into pBS (Stratagene). Clones were then screened for the presence of tissue specific DNase I hypersensitive sites. One probe detected a DNAse I hypersensitive site that exhibited a cell-specific pattern. The clone was sequenced and its location within the *Nr5a1* locus was determined.

2.6 Cell transformation

Luciferase reporter gene assays for BDNF using Fugene

PC-12 rat pheochromocytoma cells were plated at a density of 50,000 cells/24 well plate. Transfections were performed using 0.100 µg/well of promoter construct and 0.010 µg/well of mSF-1 in pCDNA3.1Zeo or empty pCDNA3.1Zeo and1.5 µl/well Fugene 6 (Roche). 48 hours after transfection, cells were harvested and luciferase levels were assayed using 20 µl of cell lysate and 100 µl of luciferase assay reagent. Results presented are +/- Standard errors of the mean (S.E.M.). Statistical significance was calculated using the Student's T test, with the variables set to paired and two tailed.

Luciferase reporter gene assays for characterization of element using calcium phosphate

Transient transfections using calcium phosphate precipitates in mouse Y1 adrenocortical tumor cells, MA-10 Leydig cells, αT3 pituitary gonadotropes, and

3T3 fibroblasts were performed as described (Sambrook and Russell, 2001), using 100-250 ng of DNA/well in 12-well plates seeded at a cell density of 50,000-100,000 cells/well. Cells were harvested 48 hrs after transfection using 400 µl of Passive Lysis Buffer (Promega), and 20 µl of lysate was assayed for luciferase activity. All transfections were performed in quadruplicate in at least 3 separate experiments**.** Results presented as the mean +/- standard error of the mean (S.E.M.). Statistical significance was calculated using the Student's T test, with the variables set to paired and two tailed.

2.7 Gel mobility shift assays

Gel mobility shift assays for the SF-1 binding sites in BDNF promoter 4 and transcription factor binding sites in the hypersensitive region

Nuclear extracts were prepared essentially as described (Shapiro et al., 1988), except that Complete Protease Inhibitor (Roche) was used instead of individual protease inhibitors. Oligonucleotides containing a G overhang were end-labeled with α^{32}P-dCTP using the Klenow fragment of DNA polymerase. Binding reactions were performed in a total volume of 20 µl containing 4 µg of nuclear extract or 6-8 µl of *in vitro* translated protein, 50,000-80,000 cpm of labeled oligo, 2 µg poly(dI-dC), in 10mM Tris pH7.8, 14% glycerol, and 1 mM DTT. Unlabeled oligonucleotide competitors and specific antibodies were pre-incubated with reaction mixture for 20 minutes on ice before the labeled oligonucleotide was added. Reactions were incubated for 20 minutes at 25ºC, subjected to electrophoresis on a 6% non-denaturing 0.25X TBE gel at 175 volts, dried, and then analyzed using a PhosphorImager. Primer sequences are listed

in Table 2-6. For electromobility shift assays (EMSA) of the SF-1 binding sites in the BDNF promoter 4, protein made from Y1 nuclear extracts or mSF-1 in pCDNA3.1Zeo and the TnT coupled reticulocyte lysate system (Promega) were used. For EMSA on the predicted transcription factor binding sites in the DNase I hypersensitive site, 4 ug of nuclear extract from Y1, MA-10, αT3, and 3T3 were used. Anti C-terminal PBX1 was a kind gift from Raymond MacDonald (UT Southwestern Medical Center; (Liu et al., 2001).

Table 2-6 Primers used for electromobility shift assays

BDNF-229	5'GAAGCCAGTGCAAGGCGATCAGGGATA3'
BDNF-229 Mutation	5'GAAGCCAGTGCAA**TT**CGATCAGGGATA3'
BDNF-144	5'GCTGAGCCCCCGCAAGGAAAAGGCGCGTCGT3'
BDNF-144 Mutation	5'GCTGAGCCCCCGCAA**TT**AAAAGGCGCGTCGT3'
BDNF-651	5'GCCGGAGAGGTCAGGGCGCAA3'
BDNF-651 Mutation	5'GCCGGAGA**TT**TCAGGGCGCAA3'
Pbx/Meis Gel Shift	5'GCCTCTCTCCTGGCAATCGCAGTGTTTAG3'
Pbx/Meis Gel Shiftmutated	5'GCCTCTCTCCTGGC**GG**TCGCAGTGTTTAG3'

Chapter 3 Development of a Transgenic Green Fluorescent Protein Lineage Marker for Steroidogenic Factor 1

3.1 Introduction

The orphan nuclear receptor steroidogenic factor 1 (SF-1) has emerged as an essential regulator of endocrine development and function. SF-1 is known to play essential roles at multiple levels of the reproductive axis. The first hints of these broader roles emerged from developmental analyses of SF-1 expression in mouse embryos (Hatano et al., 1994; Ingraham et al., 1994; Shen et al., 1994). In the adrenal and gonadal primordia, SF-1 was expressed from the earliest stages of organogenesis, suggesting a fundamental role in the initial differentiation of the primary steroidogenic tissues. SF-1 also was expressed in the developing pituitary primordium (Ingraham et al., 1994; Shinoda et al., 1995) and in neurons that ultimately form the ventromedial hypothalamic nucleus (VMH, (Ikeda et al., 1995; Luo et al., 1994).

Direct insights into the roles of SF-1 *in vivo* came from studies in knockout (KO) mice lacking SF-1 (Ikeda et al., 1995; Ingraham et al., 1994; Luo et al., 1994; Sadovsky et al., 1995; Shinoda et al., 1995). The SF-1 KO mice lacked adrenal glands and gonads and therefore died shortly after birth from adrenal insufficiency. They also exhibit male-to-female sex reversal of their external and internal genitalia, impaired expression of multiple markers of pituitary gonadotropes, and structural abnormalities of the VMH. These studies established essential roles of SF-1 at multiple levels of endocrine differentiation

and function, particularly with respect to reproduction. Developmental studies in SF-1 KO embryos showed that the earliest stages of gonadogenesis commenced in the absence of SF-1, but that the gonads then regressed due to apoptosis. Similarly, SF-1-expressing neurons migrated into the appropriate region of the developing diencephalon by embryonic day 17.5 (E17.5), but were no longer observed at postnatal day 1.

Because the adrenal glands, gonads, and VMH of SF-1 KO mice disappear at relatively early stages of development, it has been difficult to follow the fate of SF-1-expressing cells in SF-1 KO mice or to define the molecular basis for the loss of these cells in the absence of SF-1. We therefore used BAC transgenesis to develop a lineage marker for many cell lineages that express SF-1, providing a novel and versatile tool to study the roles of SF-1 in endocrine development and function. This chapter discusses the characterization of the SF-1/eGFP transgene and initial evaluation of this transgene as a tool for sorting cells.

3.2 Results

Generation of SF-1/eGFP Transgenic Mice

In previous studies (K. Parker, K. Morohashi, Y. Sadovsky, L. Heckert, unpublished observations), relatively short stretches of 5'-flanking region from the mouse *Nr5a1* gene failed to target reporter gene expression in transgenic mice to sites where SF-1 is expressed. These observations suggested that the proximal 5'-flanking region of SF-1 lacks element(s) that regulate expression *in vivo*. BAC transgenesis has emerged as a powerful approach to obtain position-

independent, copy number-dependent transgenic expression (Giraldo and Montoliu, 2001; Heintz, 2000). We therefore prepared a transgene that included a 50 kb *Bsi*W I-*Bsi*WI I fragment from a SF-1 BAC upstream of coding sequences for eGFP and 3'-splice/transcription termination signals from bovine growth hormone (Figure 3-1). The BAC included ~45 kb of 5'-flanking region, the untranslated first exon, the first intron, and sequences from exon 2 encoding the first five amino acids from SF-1. Following excision from the vector, this BAC fragment was microinjected into pronuclei to generate transgenic mice. One founder with the transgene inserted at a single autosomal locus unlinked to *Nr5a1* was used to generate the SF-1/eGFP transgenic line.

Figure 3-1 Strategy for Generating the SF-1/eGFP Transgene
The *upper diagram* shows the organization of the region of mouse chromosome 2 that includes the structural genes encoding SF-1 and germ cell nuclear factor (GCNF). A 50-kb *Bsi*WI-*Bsi*WI BAC fragment that includes the 3-exons of GCNF, the intergenic region (which represents the 5'-flanking region of SF-1), the untranslated first exon, first intron, and 32 nucleotides of second exon of SF-1 encoding the first five amino acids of SF-1 was digested with *Bsi*WI and placed upstream of the eGFP coding sequences. After excision from vector sequences by digestion with *Pme*I, DNA was used to prepare the SF-1/eGFP transgenic mouse line as described in Chapter 2. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society.

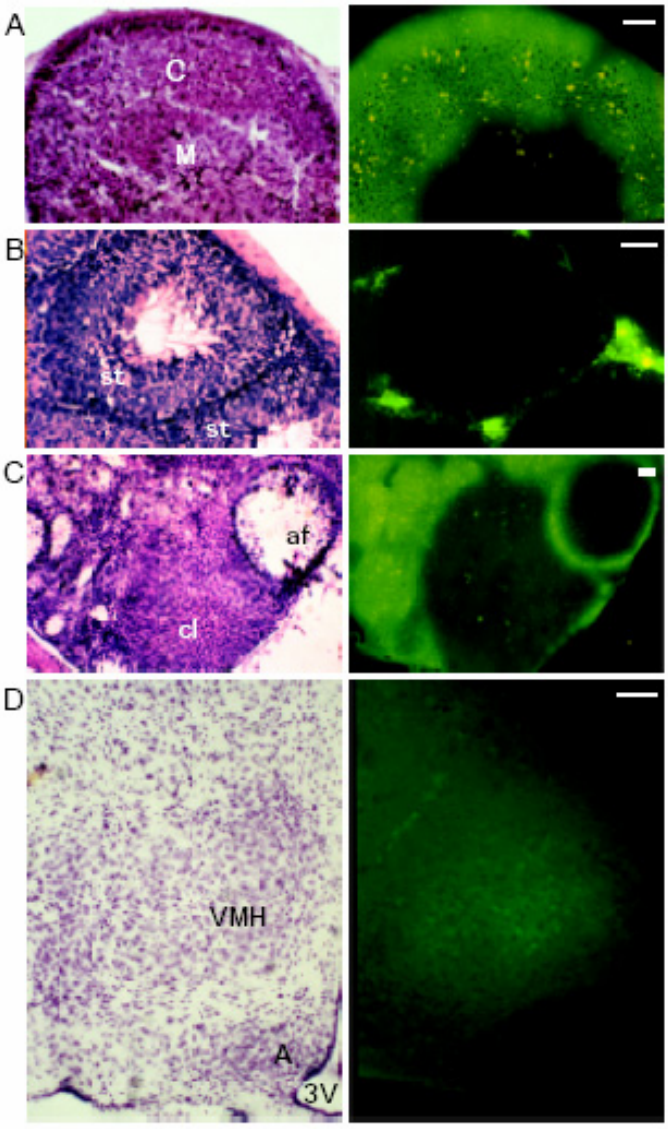


Figure 3-2 Expression of the SF-1/eGFP Transgene in Adult Mice
Tissues were harvested from adult SF-1/eGFP transgenic mice, and eGFP expression was determined as described in Chapter 2. Shown are brightfield and fluorescent photomicrographs. Panel A (testis): st, seminiferous tubule. Panel B (ovary): cl, corpus luteum; af, antral follicle. Panel C (adrenal cortex): C, cortex; M, medulla. Panel D (hypothalamus): A, arcuate nucleus; 3V, third ventricle. *Scale bars*, 100 µm. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society.

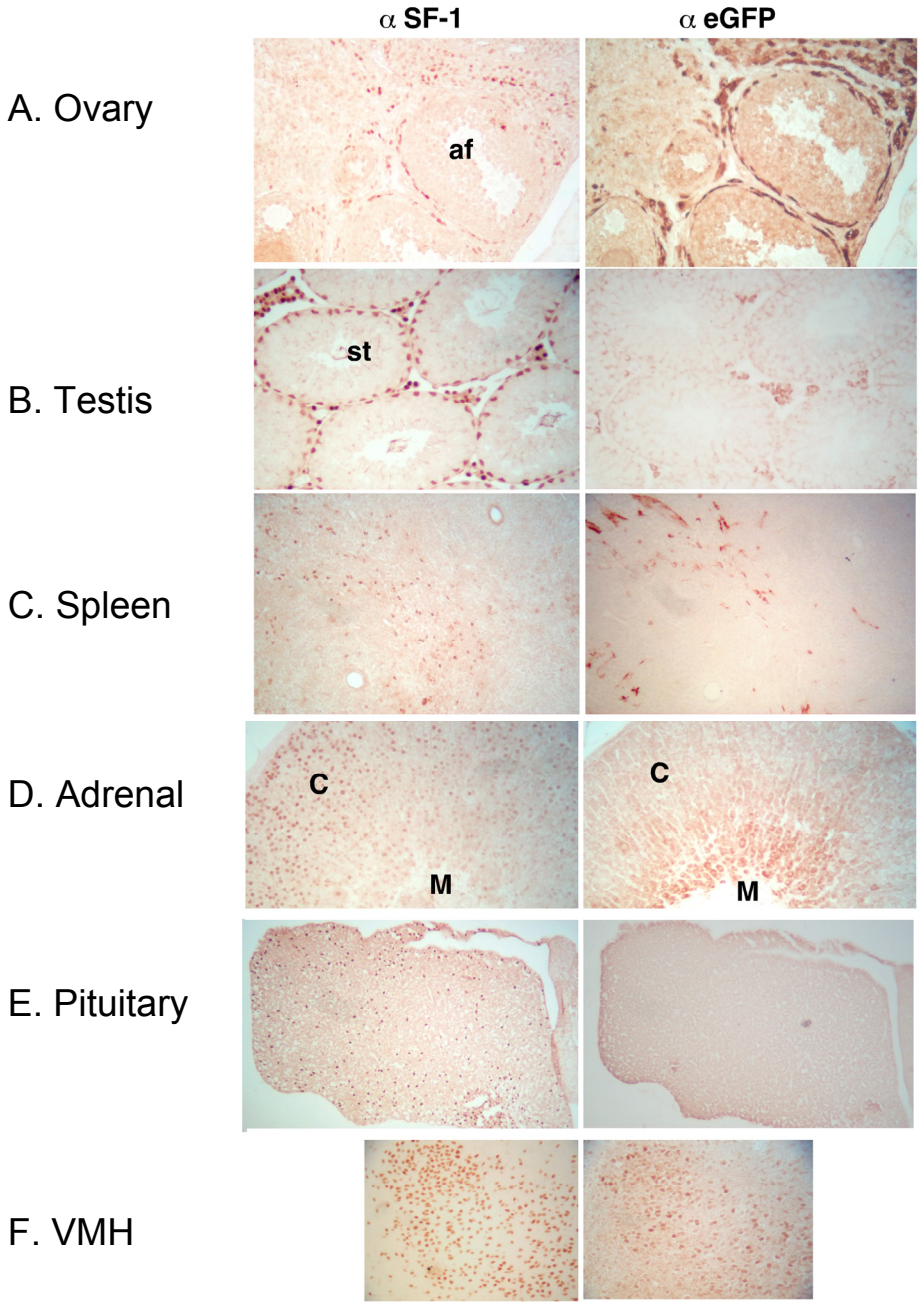


Figure 3-3 Immunohistochemical Visualization of eGFP and SF-1 Expression in Specific Cell Types

Tissues were harvested from SF-1/eGFP transgenic mice, and immunoreactive eGFP and SF-1 were visualized as described in Chapter 2. Panel A, Ovary; af, antral follicle. Panel B, Testis; st, seminiferous tubule. Panel C, Spleen. Panel D, Adrenal; C, cortex; M, medulla. Panel E, Pituitary. Panel F, VMH. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society

The SF-1/eGFP transgene targets eGFP expression to cell lineages that express SF-1.

The intrinsic fluorescence of eGFP provides a sensitive assay to determine sites where the transgenic SF-1 promoter is active. We first examined eGFP expression in adult SF-1/eGFP transgenic mice. As shown in Figure 3-2, eGFP was expressed in the interstitial regions of the testes (A), ovaries (B), adrenal cortex (C), and the VMH (D). Notably, eGFP was not expressed in corpora lutea (Figure 3-2B) or the anterior pituitary gland (data not shown). The low and high expression of the eGFP fluorescence hampered efforts to identify individual cells that expressed the SF-1/eGFP transgene. To localize eGFP expression more precisely, we performed immunohistochemical analyses with an antiserum specific for eGFP and compared the eGFP expression pattern with that of endogenous SF-1. As shown in Figure 3-3, these studies revealed striking correspondence of eGFP and SF-1 immunoreactivities in theca cells of the ovary (A), Leydig cells of the testis (B), the adrenal cortex (D), the VMH (F), and the spleen (D)—a non-endocrine organ where discrete foci of reticuloendothelial cells are known to express SF-1 (Morohashi et al., 1999). Collectively, these studies show that the SF-1/eGFP transgene in adult mice is expressed in the adrenal cortex, gonads, VMH, and spleen, suggesting that it contains sufficient regulatory information to target gene expression to most sites where SF-1 is expressed.

Although definitive conclusions are limited by our analysis of a single transgenic line, the expression patterns of SF-1 and eGFP also diverged

somewhat. SF-1 immunoreactivity was clearly observed in Sertoli cells (Figure 3-3B) and the anterior pituitary (Figure 3-3E), whereas eGFP expression was not discernable in Sertoli cells (Figure 3-3B) or in the anterior pituitary (Figure 3-3E). Although we observed no eGFP expression in either granulosa cells (Figure 3-3A) or corpora lutea, the expression patterns in these sites were not discordant, as they also did not express SF-1. The apparent absence of SF-1 and eGFP expression in granulosa cells and corpora lutea is consistent with the recent observation that these ovarian cells predominantly express the orphan nuclear receptor LRH-1 rather than SF-1 The absence of pituitary eGFP expression in SF-1/eGFP transgenic mice is consistent with the model that pituitary transcripts may arise from a distinct promoter (Kimura et al., 2000; Ninomiya et al., 1995). These results suggest that the 50 kb BAC fragment lacks element(s) required for expression in Sertoli cells and gonadotropes, although it is possible that the BAC integration site could be responsible. An important caveat regarding such conclusions is that we only analyzed a single transgenic SF- 1/eGFP line.

The developmental profile of the SF-1/eGFP transgene mirrors that of SF-1

In addition to its cell-specificity, the timing of SF-1 expression during development is precisely regulated. To determine if regulatory elements in the SF-1/eGFP transgene directed temporally-regulated expression, we examined eGFP expression in mouse embryos. At E9.0, SF-1 transcripts were first detected in the urogenital ridge, which contains cell lineages that form the gonads, adrenal cortex, and part of the kidney (Ikeda et al., 1994). Consistent with analyses of SF-1 transcripts, eGFP was expressed at E9.5 in the urogenital

ridges of both male and female embryos (Figure 3-4A, data not shown). The urogenital ridges subsequently differentiate into the indifferent gonads, which histologically are indistinguishable in males and females; at this stage (E11.5), eGFP expression again was comparable in male and female embryos (data not shown).

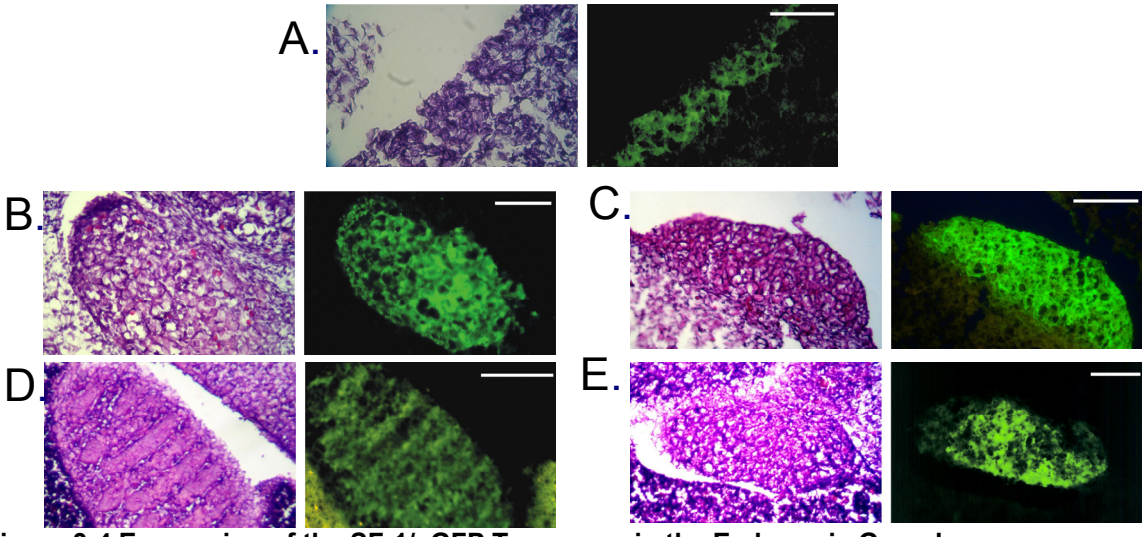


Figure 3-4 Expression of the SF-1/eGFP Transgene in the Embryonic Gonads
Tissues were harvested from SF-1/eGFP transgenic embryos at the indicated ages, and eGFP expression was determined as described in Chapter 2. Shown are brightfield (*left*) and fluorescent (*right*) photomicrographs. A, Male urogenital ridge at E9.5. B, Embryonic testis at E12.5. C, Embryonic testis at E14.5. D, Embryonic ovary at E12.5. E, Embryonic ovary at E14.5. *Scale bars*, 100 µm. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society.

At E12.5-E13, the testes organize into the testicular cords, containing Sertoli cells and primordial germ cells, and the surrounding interstitial region. At this time, eGFP was expressed by Sertoli cells within the cords and by presumptive Leydig cells in the interstitial region (Figure 3-4B). This expression pattern closely parallels previous analyses of SF-1 transcripts and is compatible with proposed essential roles of SF-1 in regulating Sertoli cell expression of anti-müllerian hormone and Leydig cell expression of the cytochrome P450 steroid

hydroxylases. As testes differentiation progressed at E14.5 (Figure 3-4D), eGFP was expressed predominantly in the interstitial region, a pattern that persisted throughout the rest of fetal development and postnatally. In fact, eGFP expression in Sertoli cells had largely disappeared by 3 weeks after birth (data not shown), and was not detected in adult mice (Figures 3-2A;3-3B).

Unlike the testes, prenatal mouse ovaries progress only to the primordial follicle stage and therefore maintain a more homogenous “ground-glass” appearance. Consistent with this relative lack of histological differentiation, eGFP was distributed throughout the embryonic ovary at both E12.5 and E14.5 (Figure 3-4C, E). Although analysis of eGFP expression by fluorescence microscopy is only semi-quantitative, the persistent eGFP expression in the ovary at this stage apparently differs from previous analyses of SF-1, which showed decreased expression in the ovaries after sexual differentiation (Hatano et al., 1994; Ikeda et al., 1994; Shen et al., 1994). Based on other transgenic analyses in which reporter genes were used to follow the activity of promoters for transcription factors (Helms and Johnson, 1998), one explanation is that the half-life of eGFP may exceed that of the SF-1 transcripts, such that eGFP persists after the cessation of SF-1 promoter activity. Alternatively, the 50 kb of SF-1 regulatory sequences employed here may lack an element that normally down-regulates SF-1 expression in the embryonic ovaries.

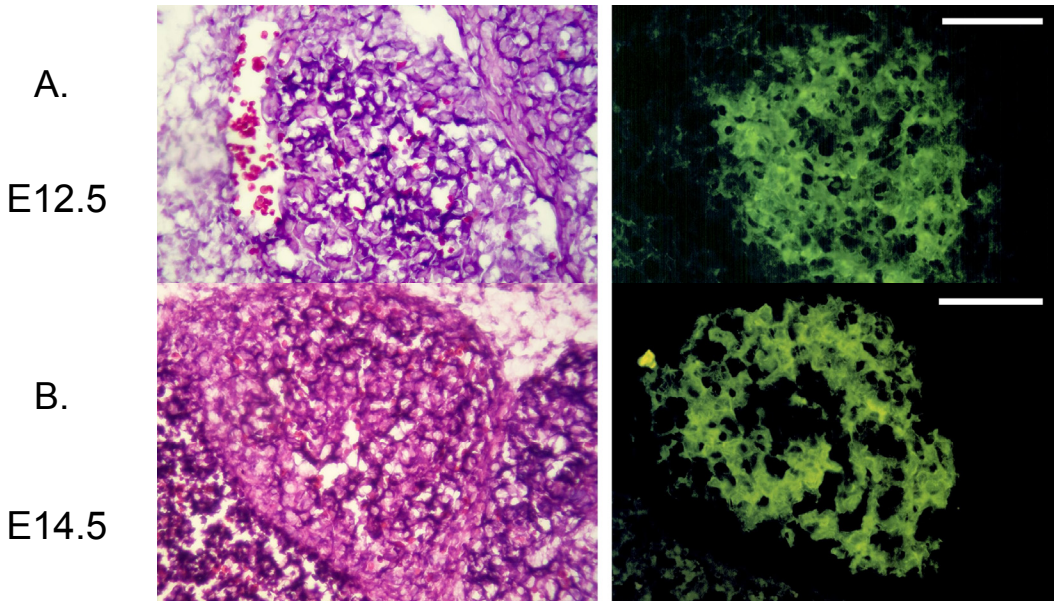


Figure 3-5 Expression of the SF-1/eGFP Transgene in the Embryonic Adrenal Glands
Tissues were harvested from SF-1/eGFP transgenic mice at the indicated ages, and eGFP expression was determined as described in Chapter 2. A, E12.5. B, E14.5. *Scale bars,* 100 μm. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society.

As shown in Figure 3-5, eGFP expression during adrenal development also correlated closely with previous studies of SF-1 expression (Ikeda et al., 1994). The adrenal primordium emerges from a common pool of adrenogonadal precursors at ~E11, forming a distinct cluster of cells (Hatano et al., 1996). Before that time, strong eGFP expression was observed in the cells that comprise the dorsomedial component of the adrenogonadal precursors, which will form the adrenal cortex. As the chromaffin cell precursors migrated into the adrenal primordium from the neural crest, eGFP expression localized predominantly to the outer region of the adrenal gland. Finally, as zonation of the adrenal cortex was established at ~E17-18, eGFP was expressed throughout all zones of the adrenal cortex (data not shown).

SF-1 transcripts have been detected at E9.5 in the ventral region of the secondary prosencephalon, the precursor of the retrochiasmatic and tuberal hypothalamus (Y. Ikeda and K. Parker, unpublished observation). When analyzed by fluorescence microscopy, this region also expressed eGFP at E9.5, albeit at a level that made it difficult to obtain a clear-cut signal on photomicrographs. This expression subsequently localized to the region of the diencephalon that comprises the VMH, correlating closely with previous analyses of SF-1 transcripts within the brain (Ikeda et al., 1995; Ikeda et al., 1994).

We also observed eGFP expression in a more caudal region of the neural floorplate immediately overlying the notochord. The significance of this finding remain to be determined, as neither SF-1 expression in this region nor spinal cord abnormalities in SF-1 KO mice have been reported. Intriguingly two different transgenes also express in this region. The first is a 674bp SF-1 LacZ reporter transgene. This short SF-1 transgenic construct did not recapitulate SF-1 expression very well, as aside from the developing spinal cord, expression was only detected in the developing gonads (Wilhelm and Englert, 2002). The second was a transgene containing 11 kb of the 5'-flanking region of Dax-1. This transgenic construct was expressed in multiple transgenic lines in a comparable region of the posterior neural tube during embryogenesis, despite the fact that Dax-1 transcripts could not be detected by RNase protection assay (Hoyle et al., 2002; Swain et al., 1998). Further studies are needed to determine if this unexpected expression directed by the promoter regions of two functionally-related orphan nuclear receptors merely reflects a confluence of artifacts or if the

transgenic promoter activity in these sites reflects endogenous expression of SF-1 and Dax-1 at levels below the limits of assay sensitivity.

In marked contrast to endogenous pituitary expression of SF-1, which commences at ~E14, eGFP was not expressed in either the pituitary primordium or developing anterior pituitary (N. Stallings, unpublished observation). Given the crucial role of SF-1 in regulating the expression of multiple genes that comprise the gonadotrope phenotype (Zhao et al., 2001), the absence of pituitary eGFP expression at any developmental stage argues that the SF-1 regulatory sequences in the SF-1/eGFP transgene lack cis-acting elements required for expression in gonadotropes. The identification of these putative elements will be an important goal for future studies.

The SF-1/eGFP transgene tracks the fate of SF-1-expressing cells in SF-1 KO mice.

Serial examination of gonadal histology in SF-1 KO mice suggested that the indifferent gonads developed in the absence of SF-1, but subsequently regressed by programmed cell death (Luo et al., 1994). Based on *in situ* hybridization analyses of a fusion transcript in the SF-1 KO mice, SF-1-expressing neurons similarly migrated into the appropriate region of the diencephalon but then disappeared between E17 and postnatal day 1(Ikeda et al., 1995) . The similar expression patterns of SF-1 and eGFP (Figure 3-3F) suggested that this transgene can track the fate of SF-1-expressing cells in SF-1 KO mice. Consistent with the relatively normal appearance of the SF-1 KO indifferent gonads at E9.5, eGFP expression at this developmental stage was

comparable in the urogenital ridges of wild-type and SF-1 KO mice (Figure 3-6A). Whereas eGFP expression was readily detected in the wild-type testis at E14.5, a distinct gonad was not present in the SF-1 KO embryo and only trace eGFP was observed in the corresponding urogenital region (Figure 3-6B). These results establish that the SF-1/eGFP transgene provides a novel tool to track the fate of cells that normally express SF-1 in the gonads of SF-1 KO mice. We have used this transgene to track the fate of VMH neurons in SF-1 KO mice (discussed in Chapter 4).

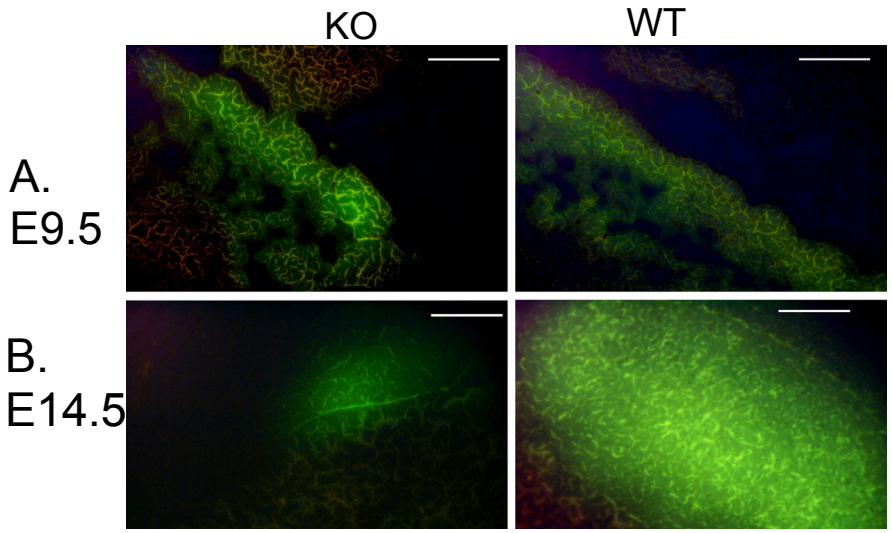


Figure 3-6 The SF-1/eGFP Transgene Tracks the Fate of SF-1-Expressing Cells in SF-1 KO Mice

Tissues were prepared from wild-type (*right panels*) and SF-1 KO (*left panels*) mice carrying the SF-1/eGFP transgene, and eGFP expression was determined as described in Chapter 2. Shown are fluorescent photomicrographs of the genital ridge or gonads at the indicated times. A, Male genital ridge at E11.5. B, Male gonad at E14.5. *Scale bars*, 100 μm. Reprinted with permission from Molecular Endocrinology 16:2360-2370.

Fluorescence-activated cell sorting selectively enriches SF-1-expressing cells.

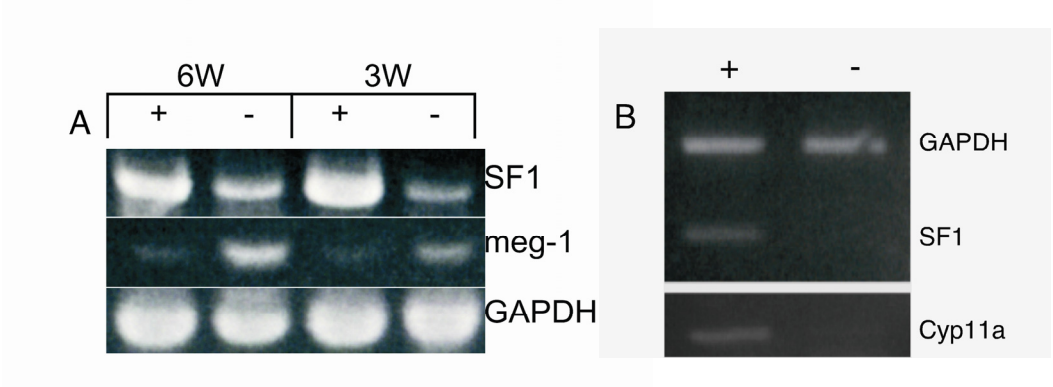


Figure 3-7 FACS Enrichment of eGFP-Positive (+) and eGFP Negative (-) Cells
Testes (3 wk and 6 wk of age) (A) and adrenal glands (newborn) (B) from SF-1/eGFP transgenic mice were dissected,pooled, and digested with collagenase to produce single cell suspensions as described in Chapter 2. Cells were resolved into positive and negative pools by FACS, and RNA samples from the positive and negative pools were used in semiquantitative RT-PCR assays to detect SF-1, cholesterol side chain cleavage enzyme, Meg-1, and glyceraldehyde phosphate dehydrogenase as described in Chapter 2. Reprinted with permission from Molecular Endocrinology 16:2360-2370. Copyright 2002, The Endocrine Society.

Previous studies with other eGFP transgenes have used fluorescence-activated cell sorting (FACS) to segregate eGFP-positive and eGFP-negative cells (Hadjantonakis and Nagy, 2000; Motoike et al., 2000). To illustrate the utility of the SF-1/eGFP transgene for similar studies, testes and adrenal glands from SF-1/eGFP transgenic mice were harvested, digested to produce single cell suspensions, and resolved into eGFP-positive and eGFP-negative populations by FACS as described in Chapter 2. To assess the success of the purification, we compared gene expression profiles in the positive and negative pools. As expected (Figure 3-7A), FACS of the testes cell population positively enriched for SF-1, while diminishing considerably transcripts for the post-meiotic germ cell marker Meg-1 (top). Similarly, the eGFP-positive pool of adrenal cells was

enriched for the expression of both SF-1 and its target gene cholesterol side-chain cleavage enzyme (Figure 3-7B). These results validate the utility of the SF-1/eGFP transgene to enrich distinct populations of cells that express or do not express SF-1 from complex tissues.

3.3 Discussion

In this chapter, we discussed the use of BAC transgenesis to place the eGFP reporter gene under the control of regulatory sequences from the *Nr5a1* locus encoding SF-1, thereby targeting eGFP to many sites that express SF-1. An important caveat is that we have assessed only one transgenic line, such that the observed expression pattern may result, in part, from the specific integration site. It is possible that observed expression patterns from transgenes is due to the integration site of the transgene and expression patterns can vary from different founder mice (Heintz, 2000). Despite this limitation, there are striking parallels between the tissue-specific and developmental profiles of eGFP expression defined here and previous studies examining the expression of SF-1 transcripts and/or protein. Finally, irrespective of the molecular mechanisms responsible for its expression, the SF-1/eGFP transgene described here will permit selective cell enrichment from many SF-1-expressing tissues (e.g., the adrenal cortex, Leydig/theca cells, and VMH neurons) as well as developmental lineage tracing in these sites, thus providing a versatile new tool for studying the roles of SF-1.

Despite considerable effort, however, the molecular basis for the tissue-specific expression of SF-1 remains poorly understood. The lack of success in

previous transgenic targeting studies suggests that the proximal promoter of the *Nr5a1* gene lacks an enhancer element necessary for high-level expression *in vivo*. To the extent that the expression of the SF-1/eGFP BAC transgene described here accurately mirrors expression of the endogenous gene, the high levels of eGFP expression in multiple cell lineages suggests that the 50 kb fragment includes one or more regulatory elements that are lacking in the proximal promoter regions used previously. The 50 kb region that suffices to target gene expression to the appropriate sites *in vivo* provides a point of departure to map specific elements that regulate the cell-specific and developmental expression of SF-1. Presumably, additional sequences are required for transgenic expression of SF-1 in gonadotropes and Sertoli cells, which exhibit a distinct dichotomy between the clear-cut expression of SF-1 and the absence of eGFP (Figure 3-3B,E). A search for novel regulatory regions of SF-1 is discussed in Chapter 5.

In addition to cell fate mapping, the SF-1/eGFP transgene has considerable potential utility for resolving SF-1-positive and SF-1-negative cells from heterogeneous mixtures such as the primary steroidogenic organs and the hypothalamus. Support for this approach is provided by our successful enrichment of SF-1-positive cells from testes and adrenal glands (Figure 3-8). The ability to isolate SF-1-expressing cells from complex structures such as the VMH or urogenital ridge should markedly facilitate approaches such as DNA chip microarrays or subtractive cloning to identify novel SF-1 target genes at different

sites of expression. Experiments using this approach are discussed in the next chapter.

In summary, this chapter describes the first successful transgenic targeting of a reporter gene with regulatory sequences from the mouse *Nr5a1* gene encoding SF-1 to the adrenal glands, gonads, and VMH. Using this transgene, we track the fate of cells that normally express SF-1 in SF-1 KO gonads and demonstrate the use of the SF-1/eGFP marker to select SF-1-expressing cells from single cell suspensions derived from the adrenal cortex. This SF-1/eGFP transgene provides novel insights into the regulation of SF-1 and will be an invaluable resource to expand our understanding of the multiple roles of SF-1 in endocrine development and function.

Chapter 4 Microarray profiling of developing VMH neurons to identify potential SF-1 target genes

4.1 Introduction

Neuronal Migration

Neuronal migration has been well studied in the formation of structures that are derived from the telencephalon (Marin and Rubenstein, 2003). Although gene families have been implicated in migration in other brain regions (Nikolic, 2004; Piper and Little, 2003), this knowledge has not been directly applied to the study of the hypothalamus. One gene whose mutation disrupts the formation of certain hypothalamic nuclei is Orthopedia. The disruption of hypothalamic development by the absence of Orthopedia includes reduced cell proliferation, abnormal migration, and problems with terminal differentiation in the paraventricular, arcuate, and supraoptic nuclei (Acampora et al., 1999). Cellular markers of these structures remain, but are aberrantly located. A similar case is seen with the KO of Steroidogenic Factor 1 (SF-1). SF-1 KO mice have a disruption in the formation of the ventromedial hypothalamic nucleus (VMH), but markers of this cell population are still present, albeit with altered location (Davis et al., 2004; Dellovade et al., 2000). Several other genes that are expressed in the hypothalamus have been implicated in cell migration in the context of other neuronal structures though analysis of defects in KO mouse models. These genes include distalless 1 (Dlx1), tenascin-c (Ten-c), and forkhead box g1 (Foxg1; BF-1).

VMH Development and SF-1

The VMH first emerges as a discrete nucleus at E15.5, but the cells that form this nucleus originate with the initial formation of the diencephalon at E9.5. The VMH has been implicated in energy homeostasis and female reproductive behavior through the use of cell ablation experiments and more recently through the use of genetically modified mouse models (Elmquist et al., 1999; Horvath and Diano, 2004; Kalra et al., 1999).

Mice lacking SF-1 do not develop the ventromedial hypothalamic nucleus (VMH) (Ikeda et al., 1995; Shinoda et al., 1995). Further studies have characterized the mislocalization of immunoreactive cell populations within the hypothalamus that are disrupted by the absence of SF-1. These studies have demonstrated the mislocalization of glutamic acid decarboxylase (GAD67), estrogen receptor α (ERα), neuropetide Y (NPY), galanin, islet-1(isl-1), NKX2.1, and brain-derived neurotrophic factor (BDNF) (Davis et al., 2004; Dellovade et al., 2000; Tran et al., 2003). Two hypothesis have been proposed for the fate of SF-1 KO neurons: incomplete terminal differentiation of the neurons (Tran et al., 2003) or altered cell migration of the developing cells (Davis et al., 2004). We have used the SF-1/eGFP transgene to locate the SF-1 KO neurons in the hypothalamus and to characterize these cells. These neurons do not die, but are rather scattered and do not form the distinct structure identified as the VMH (Davis et al., 2004).

Identification of target genes of SF-1 in the developing VMH

The purpose of this study was to identify novel SF-1 target genes in the VMH. In previous experiments we showed that it is possible to sort the eGFP/SF-1 cells from the surrounding cells and that expression of the transgene is maintained in KO embryos. We used this sorting ability, coupled with array analysis, Q-PCR, and *in situ* hybridization to identify potential novel target genes of SF-1. Embryonic day 16.5 was chosen, as the cells are still developing and migrating to their proper position in the VMH. We identified several genes that have decreased expression in the VMH of the SF-1 KO mouse, including-brain-derived neurotrophic factor (BDNF), tenascin C (Ten-c), short stature homeodomain 2 (Shox2;Prx3), distalless 1 (Dlx1), disabled 2 (Dab2), Cerebellin 1 (Cbln), Odd oz (odz2;Ten-M2) and forkhead box g1 (Foxg1; brain factor 1 or BF1).

4.2 Results

Distribution, not number, of eGFP cells is altered in the hypothalamus of SF-1 KO mice

Previous efforts to determine the fate of VMH neurons in the SF-1 KO mice used a fusion transcript encoded by the SF-1 KO allele to follow SF-1-expressing neurons using *in situ* hybridization (Ikeda et al., 1995; Tran et al., 2003). To provide a more versatile means to trace these cells, the SF-1/eGFP transgene was used to track the fate of these cells. The distribution of eGFP-positive cells is altered in the hypothalamus of SF-1 KO mice. Cells that normally localize solely within the VMH are scattered more widely in adjacent regions of the hypothalamus of the SF-1 KO

mice (Figure 4-1, A, B). For a more thorough discussion of the mislocalization of the eGFP immunoreactive cells, see Davis, et al. Journal of Neurobiology, 60: 424-436.

Counting of the eGFP immunoreactive cells in serial sections throughout the VMH showed that the number of cells is unchanged in the SF-1 KO mice versus the SF-1 +/+ mice. For each brain, every section through the VMH with eGFP signal was analyzed. Every other section was counted and the other sections were used for immunohistochemistry. We estimate that we counted approximately 50% of the total number of eGFP-positive cells in these assays. The numbers of counted cells were SF-1 +/+ 990.3±194.4, n=3 and SF-1 KO 865±227.7, n=3 (data not shown, (Davis et al., 2004)). As an additional method of counting cells, timed matings of SF-1 +/- female and eGFP+, SF-1 +/- male mice were used to generate samples for FACS of the developing hypothalamus. Embryos were dissected from the mother at E16.5 and each embryo was used to prepare separate single cell suspensions. Each single cell was analyzed for eGFP expression by FACS and positive samples were sorted. Numbers of positive cells for each sample was recorded. Genotyping for SF-1 was then done for each sorted sample. In those analyses, the number of GFP+ cells in the SF-1 +/+ mice was not significantly different from that of SF-1 KO mice. The SF-1 +/+ mice had 3263±299, n=8 and SF-1 KO mice had 3196±442, n=4. The numbers of neurons estimated by the cell counts and FACS agree relatively closely, especially since it is likely that the sensitivity of the FACS (~3200 eGFP-positive cells/ mouse) is greater than that of the immunohistochemistry and cell counting (~2000 eGFP-positive cells/mouse).

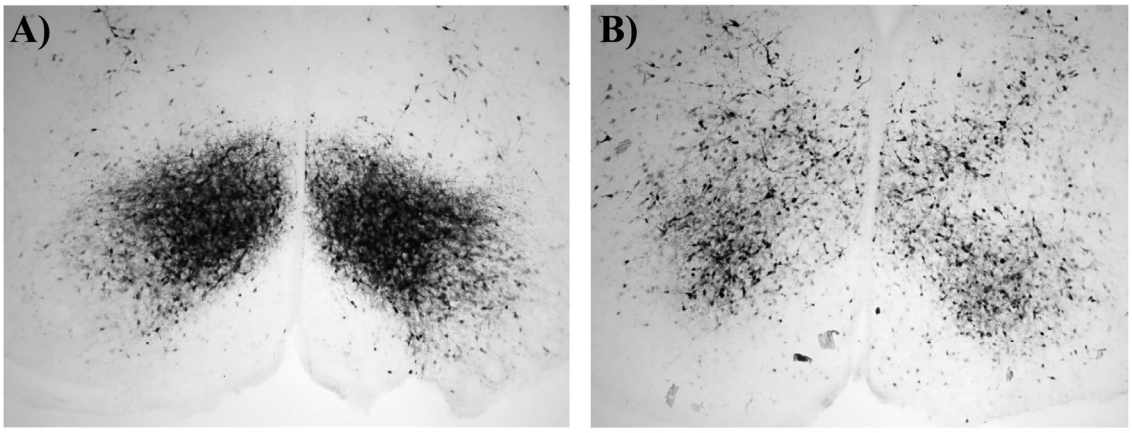


Figure 4-1 Cell position, but not number, is altered in SF-1 KO mice.
The absence of SF-1 changes the location of cells expressing a SF-1/enhanced green fluorescent protein (eGFP) transgene. Immunoreactive eGFP was used to examine the location of presumptive SF-1-expressing cells that are usually found in the ventromedial nucleus of the hypothalamus A. VMH from wild type mouse. B. VMH from knockout mouse

FACS enrichment

A total of 343 embryos at E16.5 were dissected from 60 SF-1 +/- female mice which had been timed mated with eGFP+/SF-1 +/- male mice. These embryos were prepared individually as single cell suspensions. Of these 343 embryos, 168 were found to be eGFP-positive embryos (49%) and were sorted. The resulting SF-1 and Sry (a marker of the Y chromosome) genotypes for each of the sorted embryos are detailed in Table 4-2. The observed genotypes do not significantly differ from the expected distribution as measured by the Chi-square test. Once a determination on what the cells would be used for (Microarray or QRT-PCR) was made, total RNA was pooled to establish pools with similar starting numbers of eGFP-positive cells.

Table 4-1 SF-1 genotype and chromosomal sex for eGFP positive sorted E16.5 embryos

Genotype (eGFP+)	SF-1 +/+ Male	SF-1 +/+ Female	SF-1 +/- Male	SF-1 +/- Female	SF-1 -/- Male	SF-1 -/- Female
Number	25	24	44	39	22	14
Percent	14.8	14.2	26.2	23.2	13.1	8.3

Microarray analysis reveals potential novel target genes for SF-1

Three separate hybridizations were performed for WT and KO samples using the Affymetrix U74A chips. One hybridization using a WT pool and KO pool was performed on the Affymetrix mouse 430 chip. Gene traffic V3.2, using robust multi chip analysis (RMA), was used calculate the values shown in Table 4-3. A baseline was created in the Genetraffic software program from the WT arrays. The KO samples were then compared to the wildtype samples. Downregulated genes are those whose expression is decreased in the developing VMH of the KO embryos compared to the value of the WT embryos. Upregulated genes are those whose expression is increased in the developing VMH in the KO embryos in comparison to the WT embryos. After the data were processed from the U74A arrays, 118 probe sets were downregulated >1 fold and 25 probe sets were upregulated >1 fold. From the mouse 430 array, 1131 probe sets were downregulated >1 fold and 314 probe sets were upregulated >1 fold. No further analysis was done on any of the upregulated genes, as the current study was focused on target genes of SF-1. However, these results could be evaluated in the future to help provide additional insight into the development of VMH.

Table 4-2 Upregulated and downregulated genes on Affymetrix U74A and 430 arrays

	Down > 4 fold	Down <4 fold>2 fold	Down<2 fold >1 fold	Up <4 fold	Up >4 fold<2fold	Up <2 fold >1 fold
U74A	0	7	111	0	4	21
430	83	283	765	0	32	282

The mouse 430 chip had many more genes in the downregulated and upregulated categories. This could be due to several factors: i) the mouse 430 chip has approximately triple the number of probe sets, ii) the increase in the number of

genes whose value is changed may be due to the single hybridization done with this chip and the experimental error inherent in the array procedure, or iii) an increase in the number of genes found to have differential values was due to the refinement and change of probe sets as the microarray technology improves. It is possible that all of these factors are involved. Also of note is the much greater fold change that is seen from the mouse 430 results compared to the values seen from the U74A hybridizations. This difference is probably due to the same factors that contribute to the increased number of changed genes seen in the mouse 430 chips in comparison to the U74A chips. Table 4-2 presents the top 40 downregulated “hits” in the U74A experiments and Table 4-3 shows the top 40 downregulated “hits” in the mouse 430 experiments. As a way of comparing the results from the two array formats, the top genes selected from the U74A arrays were compared to the values available from the mouse 430 array. Table 4-4 lists these values, with genes whose expression were evaluated by QRT-PCR highlighted. Although the values are not directly comparable since the two arrays use different probe sets, the majority of the genes listed had decreased expression.

Table 4-3 Genes with the largest fold change from the Affymetrix U74A array

Fold	Gene Name	GeneID	Accession
-2.62	hemoglobin, beta adult major chain, Hbb-b1	15129	NM_008220
-2.56	short stature homeobox 2, Shox2, Og12x, Prx3, SHOT	20429	NM_013665
-2.54	hemoglobin alpha, adult chain 1, Hba1, Hba-a1	15122	NM_008218
-2.52	cerebellin 1 precursor protein, Cbln1	12404	NM_019626
-2.46	alsin, amyotrophic lateral sclerosis 2 (juvenile) homolog (human), Als	74018	NM_028717
-2.24	SRY-box containing gene 11, Sox11	20666	NM_009234
-2.20	synuclein, alpha, NACP, Snca	20617	NM_009221
-1.92	distal-less homeobox 2, Dlx-2, Tes-1	13392	NM_010054
-1.86	Blmh, bleomycin hydrolase	104184	BC027403
-1.78	neurogenic differentiation 6, Neurod6, Atoh2, Math-2, Nex	11922	NM_009717
-1.76	RIKEN cDNA 2610042L04	67055	NM_025940
-1.76	cyclin-dependent kinase inhibitor 1C (P57), p57Kip2, Cdkn1c	12577	NM_009876
-1.74	ornithine decarboxylase antizyme inhibitor, Oazin	54375	NM_018745
-1.74	AI842065	105624	BU593262
-1.74	tenascin C, Hxb, Tnc	21923	NM_011607
-1.70	kit ligand, Mgf, grizzle-bell, SF, Steel factor	17311	NM_013598
-1.68	brain derived neurotrophic factor, BDNF	12064	NM_007540
-1.68	seizure related gene 6, Sez6	20370	NM_021286
-1.66	mesoderm specific transcript, Mest, Peg1	17294	NM_008590
-1.54	hypothetical protein DKFZp564K0822	232023	NM_146168
-1.52	maternally expressed gene 3, Meg3	17263	NM_144513
-1.52	phosphofructokinase, platelet, Pfkp, PFK-C	56421	NM_019703
-1.50	RAS p21 protein activator 4	100923	NM_133914
-1.48	RIKEN cDNA 2610042L04	67055	NM_025940
-1.48	LIM homeobox protein 8	16875	D49658
-1.46	distal-less homeobox 1, Dlx1	13390	NM_010053
-1.44	nucleolin, Ncl	17975	NM_010880
-1.44	hemoglobin, beta adult major chain	15129	NM_008220
-1.44	AI854265	99119	BB693547
-1.40	nuclear factor I	18028	NM_008687
-1.38	vesicular inhibitory amino acid transporter, VGAT, Viaat	22348	NM_009508
-1.38	myelin transcription factor 1-like, Png-1, Pmng1, Nztf1, Myt1l	17933	NM_008666
-1.36	CD1d1 antigen	12479	NM_007639
-1.36	zinc finger protein of the cerebellum 1, Zic1	22771	NM_009573
-1.36	tubulin, beta 5, Tubb5	22154	NM_011655
-1.34	AW120767	67597	AK017901
-1.34	nuclear receptor interacting protein 1, RIP140	18184	NM_008735
-1.34	odd Oz/ten-m homolog 2, Ten-m2, Odz2, Odz3	23964	NM_011856
-1.28	forkhead box G1, BF-1, Foxg1	15228	NM_008241

Table 4-4 Genes with the largest fold change from the mouse 430 Affymetrix array

Fold	Gene Name	GeneID	Accession
-15.18	Hbb-y, hemoglobin Y, beta-like embryonic chain	15135	NM_008221
-15.08	Hbb-y, hemoglobin Y, beta-like embryonic chain	15135	NM_008221
-8.8	RIKEN cDNA 9530006B08 gene	20750	NM_009263
-8.76	Hba1, alpha 1 globin, hemoglobin alpha, adult chain 1	15122	NM_008218
-7.42	Ets1, Tpl1, E26 avian leukemia oncogene 1, 5' domain	23871	NM_011808
-7.4	Pdgfrb, platelet derived growth factor receptor	18596	NM_008809
-7.24	Hbb-y, hemoglobin Y, beta-like embryonic chain	15135	NM_008221
-6.98	Mip2, Scyb2, Cxcl2,chemokine (C-X-C motif) ligand 2	20310	NM_009140
-6.68	Hba1, alpha 1 globin, hemoglobin alpha, adult chain 1	15122	NM_008218
-6.6	Esm1, endothelial cell-specific molecule 1	71690	NM_023612
-6.4	Hba1, alpha 1 globin, hemoglobin alpha, adult chain 1	15122	NM_008218
-6.28	Zic3, bent tail, zinc finger protein of the cerebellum 3	22773	NM_009575
-6.24	Opr, Zic5, odd-paired related, zinc finger protein of the cerebellum 5	65100	NM_022987
-6.2	Emp1, epithelial membrane protein 1	13730	NM_010128
-6.16	Math4A, neurogenin2, Atoh4, ngn-2, neurogenin 2	11924	NM_009718
-6.12	Rgs5, regulator of G-protein signaling 5	19737	NM_009063
-5.92	F13a, coagulation factor XIII, alpha subunit	74145	NM_028784
-5.86	Col3a-1, procollagen, type III, alpha 1	12825	NM_009930
-5.48	Ets1, E26 avian leukemia oncogene 1, 5' domain	23871	NM_011808
-5.38	C1qb, complement component 1, q subcomponent, beta polypeptide	12260	NM_009777
-5.38	Cfh, Mud-1, Sas1, complement component factor h	12628	NM_009888
-5.3	Tcfap2b, transcription factor AP-2 beta	21419	NM_009334
-5.18	Cfh, Mud-1, Sas1, complement component factor h	12628	NM_009888
-5.16	Agtrl1, apelin receptor, angiotensin receptor-like 1	23796	NM_011784
-5.14	Nid1, nidogen-1, entactin 1	18073	NM_010917
-5.14	Lamb1-1, laminin B1 subunit 1	16777	NM_008482
-5.14	Scyb12, Cxcl12, pre-B-cell growth-stimulating factor, chemokine (C-X-C motif) ligand 12	20315	NM_013655
-5.08	RIKEN cDNA 4631408O11 gene	66693	NA
-5.04	Laptm5, lysosomal-associated protein transmembrane 5	16792	NM_010686
-5.02	Tmvcf, Cldn5, claudin 5, transmembrane protein deleted in VCFS	12741	NM_013805
-5.02	Hexb, hexosaminidase B	15212	NM_010422
-4.92	Slc39a8, solute carrier family 39 (metal ion transporter), member 8	67547	NM_026228
-4.9	Scyb12, Cxcl12, pre-B-cell growth-stimulating factor, chemokine (C-X-C motif) ligand 12	20315	NM_013655
-4.86	Col4a1, procollagen, type IV, alpha 1	12826	NM_009931
-4.84	Alas2, erythroid-specific ALAS	11656	NM_009653
-4.8	CL-P1, Colec12, SRCL, cara4, collectin sub-family member 12	140792	NM_130449
-4.8	vitronectin, Vn, Vtn	22370	NM_011707
-4.8	Ae, Slc4a1, Empb3, erythrocyte membrane protein band 3, solute carrier family 4 (anion exchanger), member 1	20533	NM_011403
-4.76	Mail-pending, molecule possessing ankyrin-repeats induced by lipopolysaccharide	80859	NM_030612
-4.7	Clybl, Clb, citrate lyase beta like	69634	NM_029556
-4.7	Lamb1-1, laminin B1 subunit 1	16777	NM_008482
-4.68	Col3a-1, procollagen, type III, alpha 1	12825	NM_009930
-4.64	RIKEN cDNA 2310046G15 gene	76453	NM_029614
-4.6	beta2-microglobulin, Ly-m11, beta-2 microglobulin	12010	NM_009735
-4.56	Olf-1, Ebf1, early B-cell factor 1	13591	NM_007897
-4.54	Scyb4, Cxcl4, chemokine (C-X-C motif) ligand 4	56744	NM_019932
-4.52	Tnc, tenascin C	21923	NM_011607

Table 4-5 Top genes from U74A array with mouse 430 array values for comparison

Gene Name	U74A	Mouse 430 Probe Set 1	Mouse 430 Probe Set 2	Mouse 430 Probe Set 3
hemoglobin, beta adult major chain, Hbb-b1	-2.62	-5.06		
short stature homeobox 2, Shox2, Og12x, Prx3, SHOT	-2.56	-0.4		
hemoglobin alpha, adult chain 1, Hba1, Hba-a1	-2.54	-8.76	-6.68	-6.4
cerebellin 1 precursor protein, Cbln1	-2.52	-3.32	-2.56	-2.4
alsin, amyotrophic lateral sclerosis 2 (juvenile) homolog (human), Als	-2.46	-2.64	-3.74	
SRY-box containing gene 11, Sox11	-2.24	1.68		
synuclein, alpha, NACP, Snca	-2.2	-0.58	0.14	-0.38
distal-less homeobox 2, Dlx-2, Tes-1	-1.92	-1.66		
Blmh, bleomycin hydrolase	-1.86	0.02	0.18	
neurogenic differentiation 6, Neurod6, Atoh2, Math-2, Nex	-1.78	-3.44		
RIKEN cDNA 2610042L04	-1.76	0.22	0.36	0.3
cyclin-dependent kinase inhibitor 1C (P57), p57Kip2, Cdkn1c	-1.76	-2.58		
ornithine decarboxylase antizyme inhibitor, Oazin	-1.74	-0.32	-0.12	
AI842065	-1.74	-1.34		
tenascin C, Hxb, Tnc	-1.74	-4.52		
kit ligand, Mgf, grizzle-bell, SF, Steel factor	-1.7	-3.56	-1.98	-1.04
brain derived neurotrophic factor, BDNF	-1.68	-0.52	0.02	
seizure related gene 6, Sez6	-1.68	0.16	-0.84	
mesoderm specific transcript, Mest, Peg1	-1.66	-0.14		
hypothetical protein DKFZp564K0822	-1.54	0.22		
maternally expressed gene 3, Meg3	-1.52	0.8	0.74	-0.8
phosphofructokinase, platelet, Pfkp, PFK-C	-1.52	-0.3	-0.04	-0.02
RAS p21 protein activator 4	-1.5	0		
RIKEN cDNA 2610042L04	-1.48	0.22	0.36	0.3
LIM homeobox protein 8	-1.48	-3.72		
distal-less homeobox 1, Dlx1	-1.46	-1.82	-3	
nucleolin, Ncl	-1.44	-3	-1.82	
hemoglobin, beta adult major chain	-1.44	0		
AI854265	-1.44	0		
nuclear factor I	-1.4	-0.12	0.48	-0.14
vesicular inhibitory amino acid transporter, VGAT, Viaat	-1.38	-1.72	0.1	
myelin transcription factor 1-like, Png-1, Pmng1, Nztf1, Myt1l	-1.38	-1.72		
CD1d1 antigen	-1.36	-0.66	-0.74	
zinc finger protein of the cerebellum 1, Zic1	-1.36	-3.32		
tubulin, beta 5, Tubb5	-1.36	-3.32		
AW120767	-1.34			
nuclear receptor interacting protein 1, RIP140	-1.34	-0.36	-0.58	
odd Oz/ten-m homolog 2, Ten-m2, Odz2, Odz3	-1.34	-1.38		
forkhead box G1, BF-1, Foxg1	-1.28	-2.24		
Epidermal growth factor pathway substrate 15, Eps 15	-1.22	-0.24	0.14	
T-box brain gene 1, Tbr1	-1.06	-0.68		
Disabled 2, Dab2	-1			

Values are fold difference from wild type. Highlighted genes were those tested by QRT-PCR

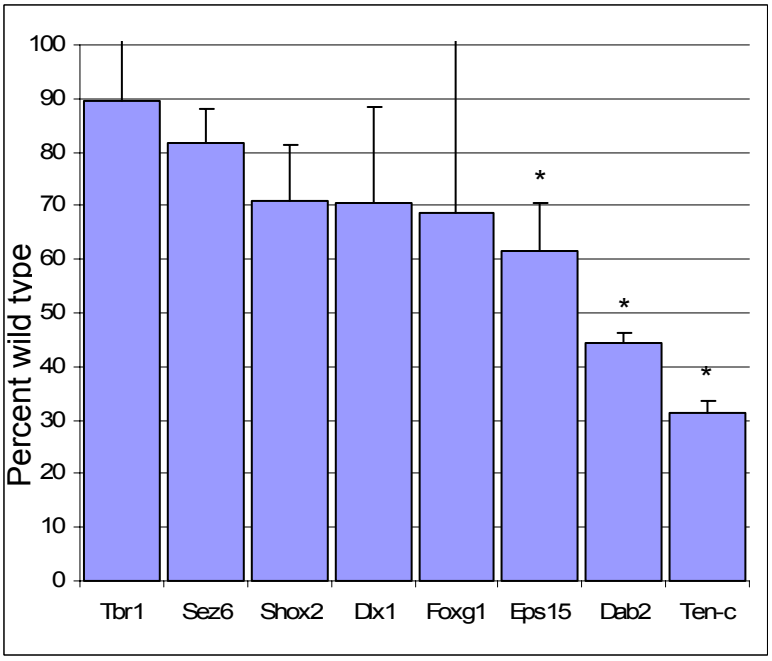
VMH Specific Markers are potential SF-1 target genes

In a separate line of experiments, laser capture microdissection (LCM) was used to isolate a set of cDNAs that are differentially expressed in nuclei of the hypothalamus. LCM was used to compare gene expression in the VMH relative to two adjacent hypothalamic regions the arcuate nucleus and dorsomedial hypothalamic nucleus. These experiments were carried out by Jeremy Segal in the laboratory of Jeff Freidman at Rockefeller University. Complete description of these collaborative experiments is available from the Journal of Neuroscience (Segal et al., 2005). Nine genes were identified and validated as markers of the VMH. One of the genes identified as a VMH marker gene was SF-1. The remaining eight VMH marker genes were examined for expression in SF-1 KO embryos. Four of the eight had decreased expression in FACS cells from SF-1 KO embryos compared to WT embryos. These four genes were cerebellin 1, Slit-3, Ten-M2, and ESTAA982708. Data comparison between this experimental approach and the SF-1 KO arrays, revealed that two of these four genes were present in the list top candidate SF-1 target genes from the Affymetrix arrays. These were cerebellin 1 and Ten-M2. Q-PCR was performed on both of these genes and *in situ* hybridization was performed on cerebellin 1. Results of these experiments are discussed below. ESTAA982708 was not present on the U74A or 430 array. Slit-3 was not present on the U74A array and had decreased expression in the mouse 430 array.

Real Time PCR confirms that some genes identified by microarray are down-regulated in SF-1 null E16.5 embryos

Real time PCR (QRT-PCR) was used to confirm that some genes identified in the array were decreased in E16.5 SF-1 KO embryos. Due to the limited amount of RNA available for the QRT-PCR, only a limited number of genes were tested, including Dab2, Cbln1, Ten-M2, Foxg1, Dlx-1, seizure related gene 6 (Sez6), epidermal growth factor pathway substrate 15 (Eps15), T-box brain gene 1 (Tbr1), and Shox2. These genes were chosen based on expression of the gene and knockout phenotypes (if known). Pools of 10,000-20,000 cells were created for the Q-PCR, and the final cDNA amount in the reactions is estimated to be 0.05-0.10 ng. Figure 4-3 shows the graph of the real time PCR results. Analysis of Cbln1 and Ten-M2 Q-PCR, using Taqman, was performed by Jeremy Segal of Rockefeller University with a cDNA pool from E16.5 embyos after the FACS enrichment (Figure 4-3B). Each of the genes analyzed using syber green Q-PCR were performed on multiple pools of cDNA, while the Taqman PCR was only performed on single pools of cDNA. Expression of Cbln, Ten-M2, Ten-c, and Dab2 was reduced to the greatest extent. Several of the other genes, such as Shox2, Dlx-1, and Eps15, also had reduced expression, albeit to a lesser extent. Although the error bars appear wide in many of the samples, the small number of repeat experiments done with each gene (2-4), plus the difficulty performing repeats within the same pool contribute to the wide variation. With the current number of replicates, changes in Eps15, Dab2, Cbln1, Ten-M2 and Ten-C are statistically significant ($p<.05$).

A.



B.

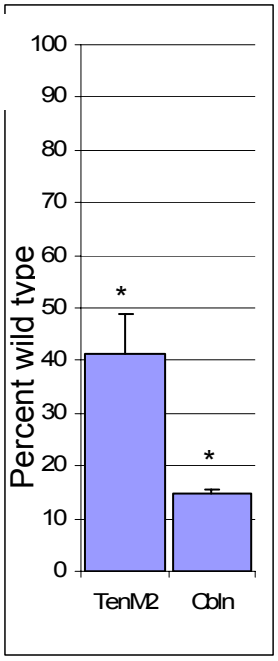


Figure 4-2 QRT-PCR Results
Expression for wild type embryos is set at 100%. Results shown are SF-1 null E16.5 embryos compared to SF-1 wild type E16.5 embryos. Wild type values are set at 100%. Error bars represent S.E.M. *=<.05
A. Syber Green
B. Taqman

In situ analysis of BDNF and Cerebellin 1

To examine the expression of BDNF and cerebellin 1 in SF-1 KO mice, a brain specific SF-1 KO was used. The SF-1 gene was ablated conditionally (Zhao et al., 2001) using a Cre transgene expressed under the control of a Nestin promoter. Although the VMH organization is not as severely disrupted in these animals as in the SF-1 null mice (Davis et al., 2004), the mice exhibit abnormalities in behavior and energy homeostasis (L. Zhao and K. Parker, personal communication). *In situ* hybridization was performed on the adult brain of conditional SF-1 KO mice. Figure 4-3 shows the dramatic decrease in the expression of BDNF and cerebellin 1 in the VMH of the tissue specific KO mice compared to the control mice.

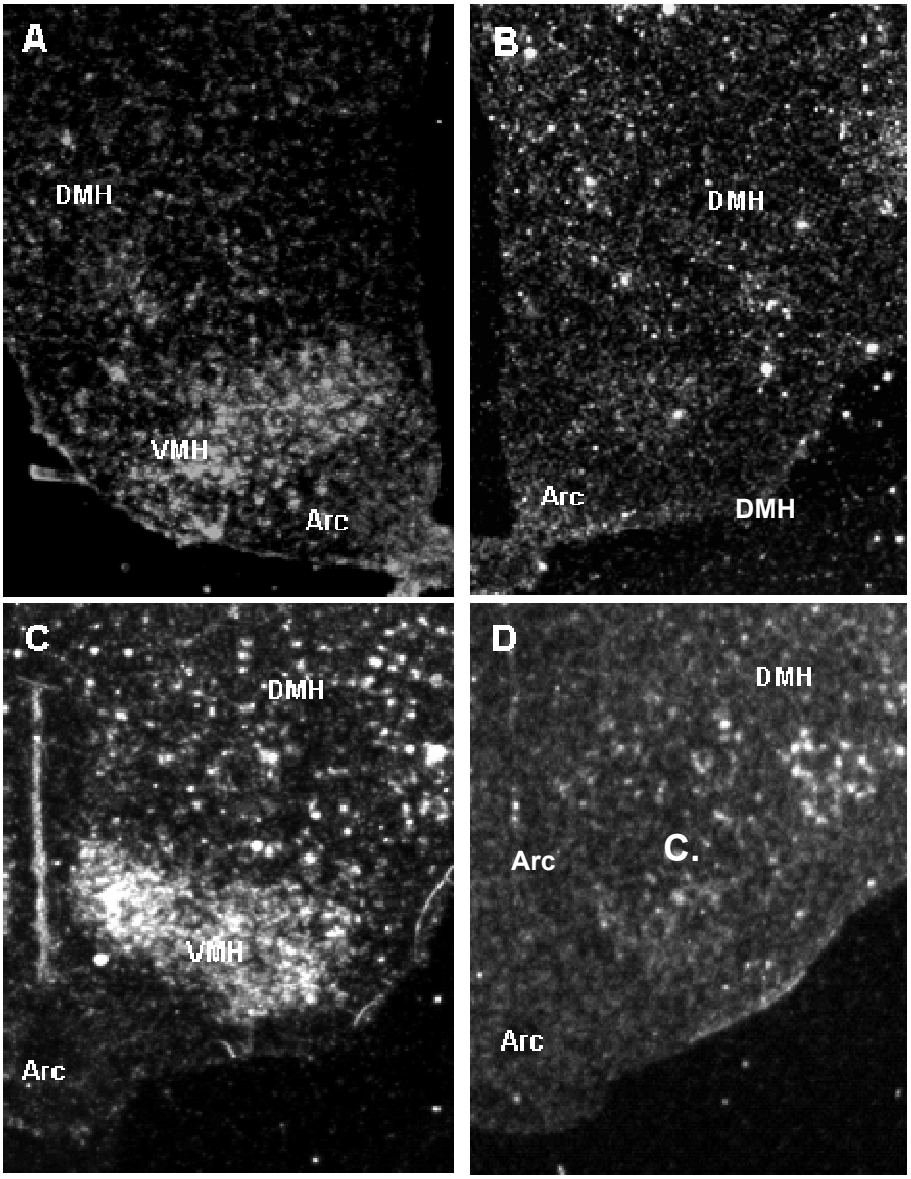


Figure 4-3 *In situ* hybridization reveals reduced expression of BDNF and Cerebellin in CNS-conditional SF-1 null mice

DMH, dorsomedial hypothalamic nucleus; Arc, arcuate nucleus; VMH, ventral medial hypothalamic nucleus

A. BDNF wild type
B. BDNF CNS-KO
C. Cerebellin wild type
D. Cerebellin CNS-KO

4.3 Discussion

Limitations of experimental methods

These experiments treat all SF-1-expressing cells as a homogenous population and do not distinguish between early and late born neurons or their different positions in the developing VMH. This is an important point because early born cells apparently have a more severe migration phenotype than later born cells (Davis et al., 2004). Expression values also cannot distinguish between direct target genes of SF-1 and genes whose expression is modified secondarily to the disrupted positional cues within the hypothalamus. Further analysis of these genes is necessary to determine whether they are direct targets of SF-1. This analysis could include promoter analysis to identify SF-1 binding sites and luciferase assays, EMSA, or chromatin immunoprecipitation to assess the ability of SF-1 to interact with these binding sites. Immunohistochemistry and *in situ* hybridization also can be employed to investigate expression of these genes in the developing and adult VMH. Another significant limitation in this analysis is the use of a single developmental time point. It is possible that genes with transient expression in the developing hypothalamus will be missed in this analysis, as well as genes with expression whose onset is after E16.5. A more thorough analysis of the developing VMH can be undertaken by using several different developmental stages. We are limited to embryonic time points, since postnatal neurons are not amenable to the FACS approach and SF-1 KO mice die very shortly after birth. Despite these limitations, many interesting potential target genes have been identified in this screen and

continuing analysis of these genes should confirm if any are *bona fide* target genes of SF-1.

Discussion of potential target genes

Several of the genes identified in the array analysis have been suggested to be involved in VMH development. Such findings include published expression data and KO mouse models.

Brain-derived neurotrophic factor

BDNF is expressed in the developing hypothalamus (Sugiyama et al., 2003). BDNF heterozygous mice and brain-specific KO mice exhibit a late onset obesity (Kernie et al., 2000, Rios et al., 2001), similar to the adrenal-rescued SF-1 KO mice (Kernie et al., 2000; Majdic et al., 2002; Rios et al., 2001). BDNF as a target gene of SF-1 is further discussed in Chapter 5.

Cbln1

Cerebellin 1 (Cbln 1) is a 16 amino acid peptide that is widely distributed in the central nervous system, adrenal, and developing testis ((Kavety and Morgan, 1998; Mazzocchi et al., 1999). Cbln 1 shares a significant homology with the globular domain of C1q and adiponectin (Hu et al., 1996; Kavety and Morgan, 1998). The similarity to adiponectin is interesting because adiponectin expression was recently shown to be controlled by LRH-1, a nuclear hormone receptor closely related to SF-1 (see Chapter 1) (Iwaki et al., 2003).

Foxg1

Foxg1 (also known as brain factor 1 or BF1) is a winged helix transcription factor. Mice null for this transcription factor have characterized defects in the formation of some structures in the dorsal telencephalon and ventral telencephalon (Hanashima et al., 2002). More specifically, the Foxg1 -/- mice have neurons that differentiate prematurely in the telencephalon (Pratt et al., 2004). A Foxg1^{lacZ} reporter transgene expresses in the ventral hypothalamus, but more specific localization of the signal has not been reported (Pratt et al., 2004).

Dab2

The disabled proteins, Dab1 and Dab2, are a family of adaptor proteins involved in cellular signaling, development, and oncogenesis (Yang et al., 2002). Dab 1 mutant mice have defects in brain formation (Lambert de Rouvroit and Goffinet, 2001). Dab2 mutants are embryonic lethal at E6.5 due to defects in endodermal cell positioning (Yang et al., 2002).

Eps15

Eps15 is expressed in the mouse brain, but no details on the hypothalamus are known (Offenhauser et al., 2000). EPS15 is thought to be involved with cellular functions connected with endocytosis, actin remodeling, and intracellular transduction of signals (Confalonieri and Di Fiore, 2002). Eps15 plays a role in clathrin-dependent endocytosis (Chen et al., 1999).

Distal less 1

Data from the Gensat database (http://www.gensat.org/index.html) (Heintz, 2004) shows that Dlx1 is expressed in the developing hypothalamus at E15.5, although the use of sagittal sections in this database makes it hard to tell exactly which nuclei are expressing the gene. Data presented in the papers characterizing the orthopedia null mice show expression of Dlx1 in what would be predicted to be the VMH, but sections are not labeled as such and figures are presented in such a way that it is difficult to use other anatomical landmarks to be sure of the zones of expression (Acampora et al., 1999).

Short stature homeobox 2

Expression of Shox2/Prx3/og12 is seen at E11 in the diencephalon and expression persists at E16.5 (Blaschke et al., 1998; Semina et al., 1998; van Schaick et al., 1997). Expression was not detected in whole adult hypothalamus by northern blot analysis (van Schaick et al., 1997). Expression was also detected in the developing gonads (Blaschke et al., 1998; Semina et al., 1998).

Tenascin C

The tenascin C knockout mouse was created in 1992 and the first papers on the knockout reported no abnormalities, despite the widespread expression of tenascin C (reviewed (Mackie and Tucker, 1999). Further analysis of these mice and a second KO line, revealed that the knockout mice have abnormal behavior (Kiernan et al., 1999) and altered gene expression in their brains (Fukamauchi et al., 1997). The altered behaviors include hyperactivity, poor swimming, abnormal

circadian rhythms, and deficits in coordination (Fukamauchi et al., 1996; Kiernan et al., 1999). The paired related homeobox transcription factors Prx1 and Prx2 are able to activate transcription of the Ten-C promoter (McKean et al., 2003) and a related protein, Shox2/Prx3, was identified in this study as being downregulated in SF-1 KO mice. Since the Prx family members have nearly identical homeodomains (van Schaick et al., 1997), it is possible that Prx3 could also bind to the promoter of ten-c and activate transcription. This suggests the possibility that the downregulation of Ten-c seen in the SF-1 KO mice could be a secondary effect versus a primary effect.

Ten-M2/Odz2

The mouse ten-m family of proteins consists of four proteins that are related to the drosophila ten-m/odz pair rule gene. The ten-m/odz family are the only pair rule proteins that are not transcription factors. The protein structure contains EGF motifs and the rest of the protein structure suggests that they act by type II transmembrane molecules (Oohashi et al., 1999). Ten-M2 is expressed in the developing diencephalon at E12.5 (Zhou et al., 2003). The other Ten-m proteins have similar, yet distinct, patterns of expression in the developing and adult mouse brain (Zhou et al., 2003)

Chapter 5 Analysis of BDNF as a target gene of Steroidogenic Factor 1

5.1 Introduction

BDNF

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of proteins. The neurotrophins are traditionally thought of as nerve survival factors. The neurotrophin family includes BDNF, nerve growth factor (NGF), NT-3, and NT-4/5. The members of the neurotrophin family bind to p75NGFR receptors, members of the tumor necrosis family, but they each also have a high-affinity receptor in the Trk (tropomyosine-related kinase) family. NGF binds to TrkA, BDNF, and NT-4 to TrkB, while NT-3 interacts mainly with TrkC and also at lower affinity with TrkB receptors. Trks are involved in many signaling pathways, including those containing Ras, the Cdc42/Rac/RhoG protein family, MAPK, PI3K, and PLC-γ (Tapia-Arancibia et al., 2004).

Genomic Organization of BDNF

The BDNF genomic organization is complex. At least 8 different transcripts of BDNF are made through the use of 4 promoters and unique 5'-exons, and one coding exon with two polyadenylation signals. The promoter and polyadenylation

signals are used independently of each other. This gene organization is diagrammed in Figure 5-1. Although there are only four promoters characterized to date, EST data deposited in Genbank suggests that there could be more transcripts. The rat and human genomic organizations are very similar to that of the mouse.

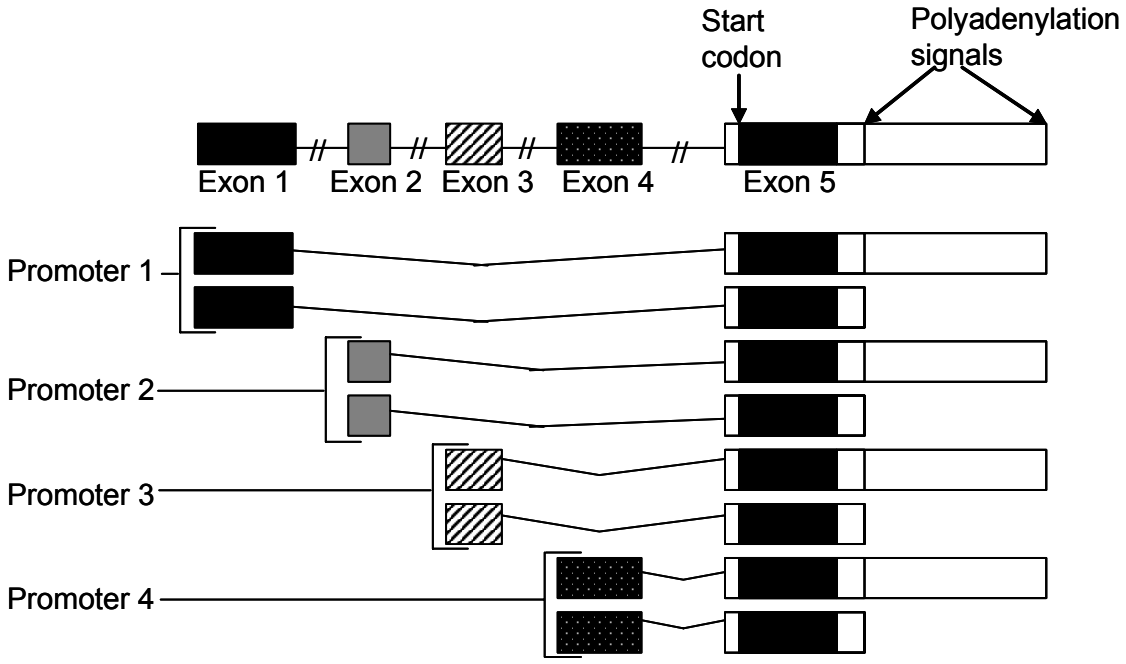


Figure 5-1 Schematic of BDNF Gene
The top diagrams the BDNF gene. Exons are represented by boxes and introns by lines. The lower diagrams show the documented alternative splice forms of BDNF

BDNF Expression

BDNF protein is widely expressed in the mouse brain, beginning early in development and continuing throughout adulthood. In the hypothalamus, it is detected in the arcuate, ventromedial, and dorsomedial nuclei (Kawamoto et al., 1996). BDNF transcripts in the VMH increase in expression from E16.5 to birth and peak at postnatal day 4, with levels falling to modest expression levels in adulthood (Sugiyama et al., 2003).

Regulation of BDNF Promoters

The various BDNF promoters respond to different stimuli and act independently of each other. For instance, promoter 1 and promoter 2 are controlled by a neuron-restrictive silencer element (Timmusk et al., 1999), act as immediate early genes (Lauterborn et al., 1996), and are upregulated by electroconvulsive seizure (Dias et al., 2003). Promoter 3 is regulated by methylation (Chen et al., 2003; Marmigere et al., 2001; Martinowich et al., 2003), promoter 2 by thyroid hormone (Koibuchi et al., 1999), and promoter 1 by calcium mediated signals through CRE and USF binding elements (Tabuchi et al., 2002). All four transcripts are regulated by glutamate, but have differential responses (Marmigere et al., 2001). Promoter 4 can be upregulated by calmodulin-dependent protein kinase II (Takeuchi et al., 2000), MAPK kinase kinase and protein kinase A (Takeuchi et al., 2002). This differential regulation of the promoters is supported by data showing that the BDNF transcripts are expressed differentially across regions of the brain (Bishop et al., 1994). In addition, the two polyadenylation signals in the coding exon are used independently (Ohara et al., 1992). This results in eight transcripts producing one identical protein.

BDNF KO

Global

Heterozygous BDNF KO mice exhibit either abnormalities in eating behavior or locomotor activity (Kernie et al., 2000). Expression of BDNF was greatly reduced

in the VMH of the heterozygous KO mice, although the mice retained some expression (Kernie et al., 2000). Dietary restriction of the heterozygous mice increases the expression of BDNF to the levels seen in wild type mice and reverses the obesity, hyperphagia, and hyperactivity phenotype seen in the heterozygous mice (Duan et al., 2003).

Tissue specific

BDNF brain specific KO mice were created using a cre transgene that deletes BDNF expression in post mitotic neurons between the second and third weeks of life (Rios et al., 2001). The BDNF conditional KO mice had increased body weight, which was statistically significant by eight weeks (Rios et al., 2001). The conditional mutants also displayed several behavioral abnormalities such as increased anxiety and stress-related hyperactivity (Rios et al., 2001).

Preliminary evidence supporting the hypothesis that SF-1 regulates BDNF activity

SF-1 knockout mice normally die shortly after birth, but the mice can be rescued through adrenal transplants from wildtype littermates (Majdic et al., 2002). These adrenal rescued SF-1 KO mice develop obesity by 8-10 weeks. This late onset obesity is very similar to that developed by the BDNF heterozygous mice (Kernie et al., 2000) and BDNF brain specific KO mice (Rios et al., 2001). In addition, it has been reported that BDNF expression, as measured through a lacZ reporter knockin, is greatly reduced in the VMH of SF-1 KO mice. These

observations led us to investigate whether BDNF is a direct target gene of SF-1 in the VMH of mice.

5.2 Results

BDNF promoter 4 contains three SF-1 binding sites

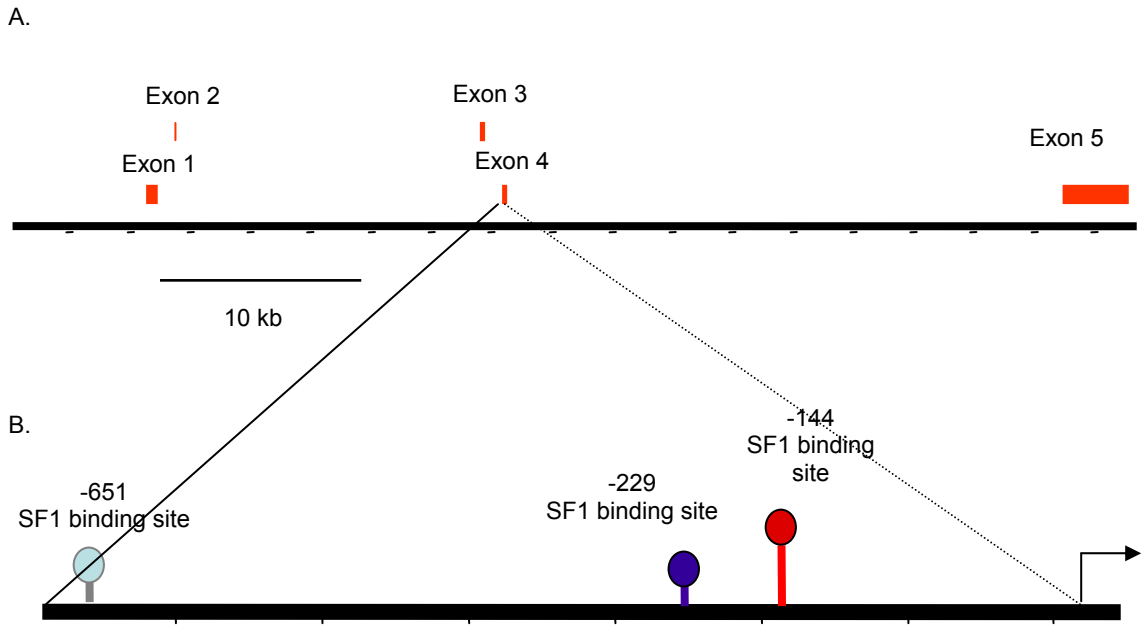


Figure 5-2 Organization of the BDNF gene and location of SF-1 binding sites
A. Genomic organization of the BDNF gene
B. Location of the three predicted SF-1 binding sites in BDNF promoter 4

Analysis of the four characterized BDNF promoters (Figure 5-2A) revealed that promoters 1, 3, and 4 had putative SF-1 binding sites. Promoter 4 had three predicted SF-1 binding sites within 700 bp upstream of the transcription start site, whereas the predicted binding sites in the other promoters were all more than 3kb from the transcription start site. For this reason, further studies focused on the

binding sites in promoter 4. Figure 5-2B shows the location of the three SF-1 binding sites at -651, -229 and -144 within BDNF promoter 4.

SF-1 can bind to all three sequences in the BDNF promoter 4

In order to analyze the ability of SF-1 protein to bind to the predicted binding sites in BDNF promoter 4, electrophoretic mobility shift assays were performed. As shown in Figure 5-3, all three predicted binding sites were able to bind *in vitro* translated SF-1 protein. Y1 nuclear extracts, which contain SF-1 protein, also formed a band that was competed using an antibody against the DNA binding domain of SF-1. Mutation of the GG of each binding site to TT abolished the binding to all three of the SF-1 binding sites, as expected since the GG of the binding site is essential for SF-1 binding. SF-1 also showed different apparent binding affinities to the sites. Site -144 bound the protein with the weakest affinity, but it also was the binding site that deviated the most from the consensus SF-1 binding site (GCAAGGAAA versus the consensus of CCAAGGTCA).

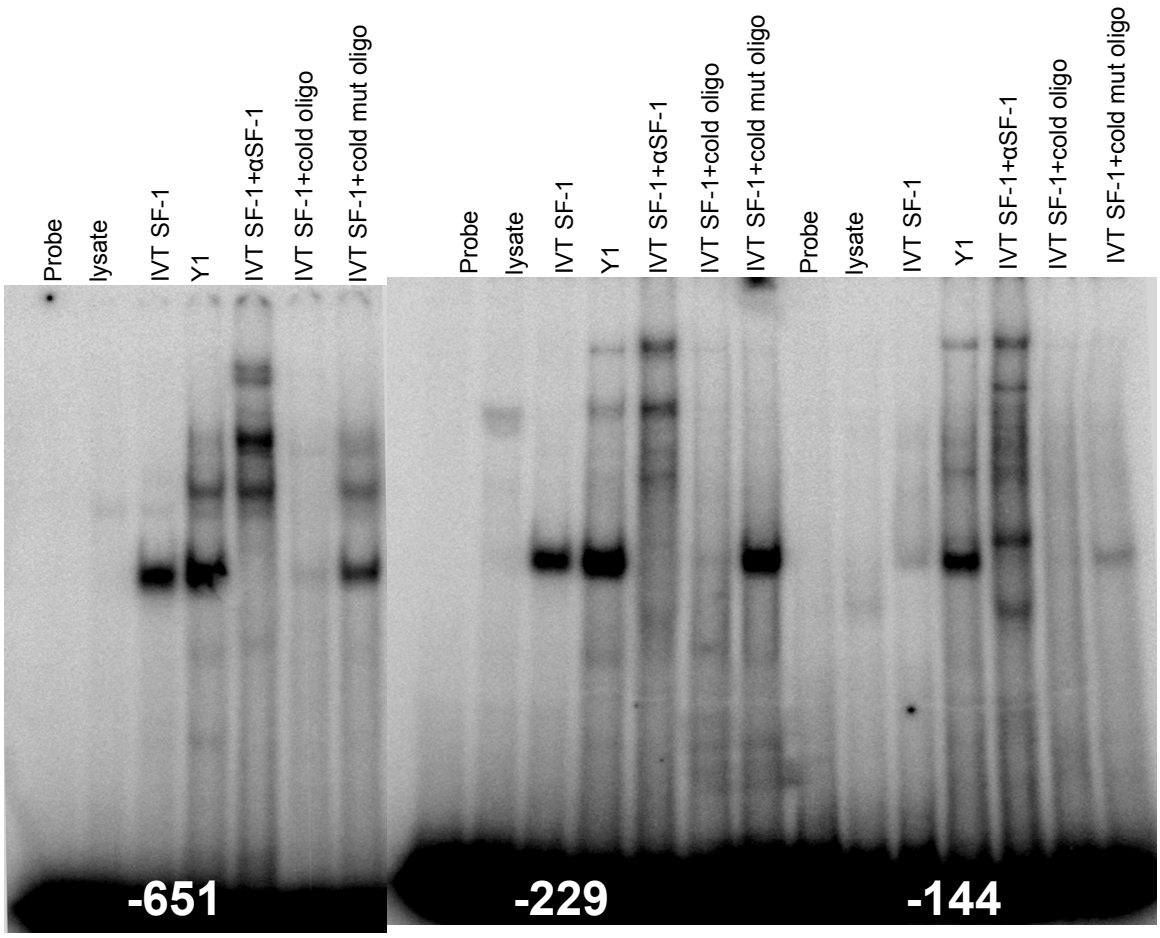


Figure 5-3 SF-1 protein is able to bind to three elements in BDNF promoter 4
EMSA was performed to test the ability of SF-1 protein to bind to the predicted sites in BDNF promoter 4. Numbers indicate location of the SF-1 binding site in BDNF promoter 4.

SF-1 can activate transcription from the BDNF promoter 4

To test the ability of SF-1 to activate the promoter of BDNF, a luciferase reporter construct was made for promoter 4. The promoter construct had activity in Y1, PC-12, αT3, SK-N-SH, and COS cells. Much less promoter activity was observed in JEG-3 cells. Next the ability of SF-1 to increase the activity of the promoter was tested. In co-transfection experiments, SF-1 increased the activity of

the promoter 1.59 fold (± .25S.E.M., p=.04, n=11; data not shown) in COS cells and 1.54 fold (± .09S.E.M., p=.000013, n=21) in PC-12 cells. The data for PC-12 cells is shown in Figure 5-4. Mutation of the binding sites did not reduce basal activity in PC-12 cells (data not shown). This is expected because PC-12 cells do not express SF-1. However, mutation of the binding sites did affect the ability to increase expression by co-transfection of SF-1, to varying degrees for each of the binding site mutations (Figure 5-4). Mutations were created that replaced the middle GG in each binding site to TT. The GG is essential for SF-1 to bind. Each one of the mutations created in the BDNF promoter 4 blocked statistically significant increases in promoter activity in SF-1 co-transfection studies (Figure 5-4).

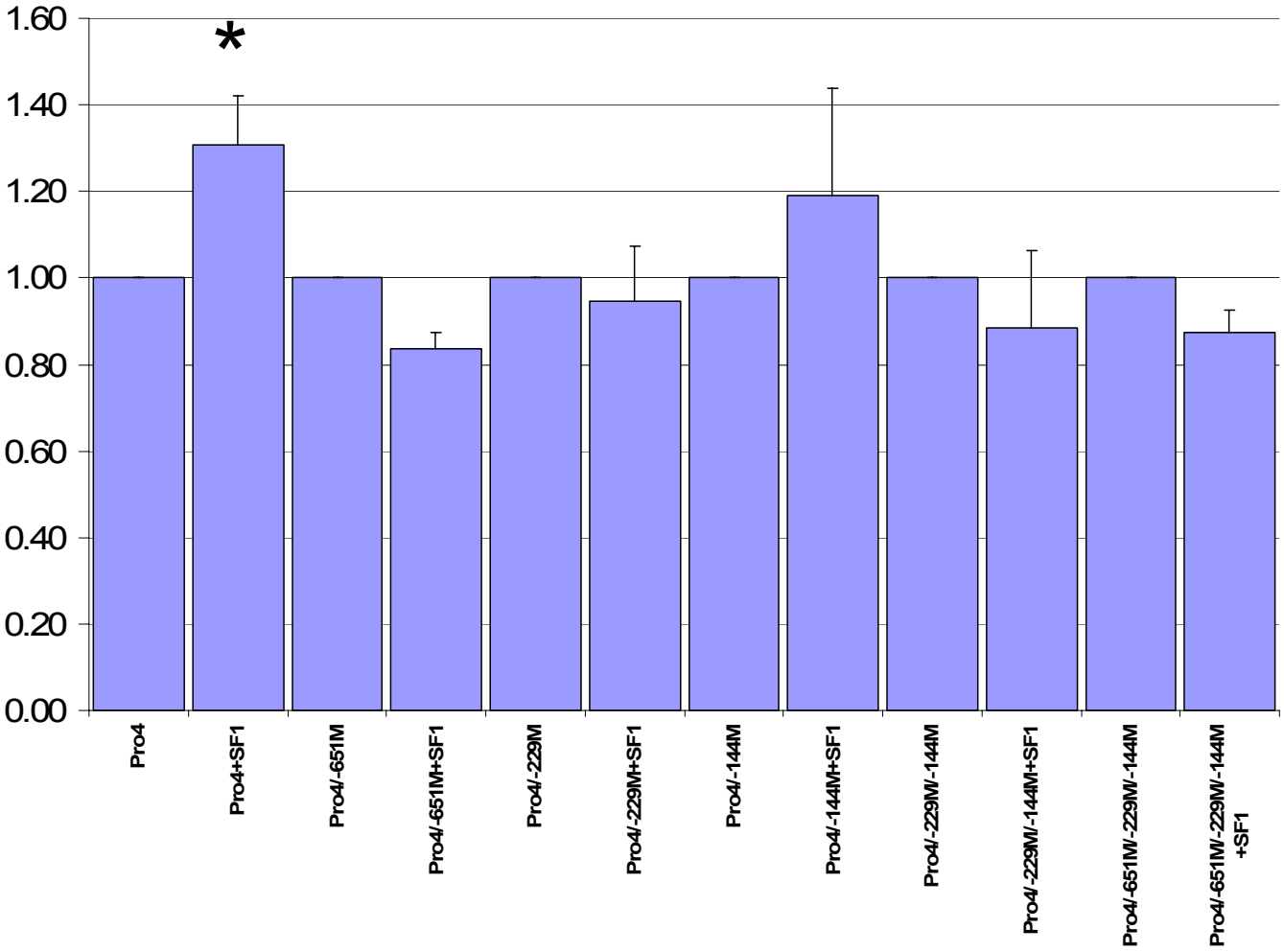


Figure 5-4 SF-1 is able to enhance the activity of Promoter 4 in PC-12 cells
Transient transfections were performed to test the ability of SF-1 to enhance the promoter activity of BDNF promoter 4. 100ng of the BDNF promoter plasmid and 10ng of the SF-1 expression plasmid were used per well in 24 well plates. Fold values are set to 1.00 for each construct. Error bars are ±S.E.M. The asterisk represents a p value of <0.001 from the student's T-test.

BDNF expression is decreased in the VMH of SF-1 null animals

BDNF expression was examined in the VMH of WT and SF-1 conditional KO mice. As shown in Figure 4-3A,B, the expression of BDNF in the VMH of SF-1 conditional KO mice was decreased as measured by *in situ* hybridization.

Expression in other brain areas was not affected (L. Zhao, personal communication). This agrees with previous results where BDNF expression is decreased in the VMH of SF-1 global KO mice (Tran et al., 2003).

5.3 Discussion

Linking SF-1 and BDNF to obesity

Adrenal rescued global SF-1 KO mice developed a late onset obesity and had a decreased activity as measure by wheel running activity (Majdic et al., 2002). The use of a brain-specific Cre recombinase to inactivate SF-1 shows that this obesity phenotype is at least partially replicated, suggesting that the obesity seen in the global knockouts can be attributed to the disorganization of the VMH (L. Zhao and K. Parker). BDNF heterozygous mice also display a similar pattern of late onset obesity (Kernie et al., 2000; Rios et al., 2001). The use of a $BDNF^{lacz}$ reporter transgene (homologous recombination was used to replace the coding sequence of BDNF with LacZ) suggested that BDNF could be a target gene of SF-1, as the reporter transgene had reduced activity in the SF-1 KO mice (Tran et al., 2003).

BDNF as a target gene of SF-1

The reduced BDNF activity in P0 SF-1 KO mice, as measured by a $BDNF^{lacz}$ reporter transgene, led us to investigate BDNF as a direct target of SF-1. Transient transfections of BDNF promoter 4 with SF-1 leads to a very modest increase in promoter activity. Despite the reduced expression of BDNF in the VMH of adult

tissue specific SF-1 knockouts, real time PCR failed to confirm that total BDNF transcripts were reduced in the developing VMH at E16.5. However, at E18.5, a developmental time point with increased BDNF expression, QRT-PCR was able to confirm a reduction of all BDNF transcripts in the SF-1 KO mice (L. Zhao, personal communication). Limitations in the amount of RNA available have prevented a transcript specific QRT-PCR, but those experiments are currently being pursued. The QRT-PCR results are important because they help answer a question about BDNF expression that arises from analysis of the *in situ* data. Since the VMH is not formed in the SF-1 global KO or nestin Cre/CNS-specific KO, it is hard to confirm that BDNF expression is really reduced, since it may only appear to be reduced due to the mispositioning of cells. The methodology employed to dissect the hypothalamus ensures that the FACS sorted cells include all of the eGFP positive cells from the hypothalamus, this ensures that cells that are not migrating to the correct position are still included from analysis of BDNF expression.

The data show that BDNF expression is reduced in the VMH of both SF-1 global and CNS-specific KO mice. Analysis of BDNF promoter 4 shows that SF-1 is able to bind to three sites within the promoter and luciferase assays show that SF-1 is able to modestly increase the expression of this promoter. Taken together, these results suggest the BDNF is a very strong candidate to be a SF-1 target gene.

Chapter 6 Identification of a Conserved Intronic Element that Regulates the Cell-selective Expression of Steroidogenic Factor 1

6.1 Introduction

In an effort to define the mechanisms that specify the temporal and spatial expression profile of SF-1, several groups have tried to define the mechanisms that regulate the promoter activity, generally focusing on transient transfection analyses of the proximal 5'-flanking region in cultured cell lines (Figure 5-1). As shown in Figure 5-1, these studies have identified several elements that regulate SF-1 expression, including an E box at –80 that binds basic helix-loop-helix (bHLH) family members (Harris and Mellon, 1998; Nomura et al., 1995); a binding site for GATA4 (Tremblay and Viger, 2001), a Sox9 site (Shen and Ingraham, 2002), a SF-1 responsive element (Nomura *et al.*, 1996), as well as other elements such as CAAT boxes and Sp1 sites (Woodson *et al.*, 1997).

In addition to the transient transfection analysis, several groups have used transgenes derived from the *Nr5a1* locus to drive expression of reporter transgenes (Stallings et al., 2002; Wilhelm and Englert, 2002) or expression of other cDNAs (Jeays-Ward et al., 2003, N. Bingham and K. Parker). In general, these efforts have failed to fully recapitulate the endogenous expression pattern of SF-1, suggesting that these transgenes lacked elements that regulate SF-1 expression *in vivo.*

An alternative strategy to define potential regulators of SF-1 expression is to identify genes whose mutation impairs SF-1 expression. Through this approach, genes proposed to regulate SF-1 expression include the zinc finger protein Wilms' tumor 1 (WT1) (Wilhelm and Englert, 2002), the homeodomain protein Lhx9 (Birk et al., 2000), the homeodomein protein Arx (Kitamura et al., 2002), and the homeodomain protein Pbx1 (Schnabel et al., 2003). Despite considerable effort, however, the mechanisms that regulate SF-1 expression in a cell- and tissue-specific manner remain poorly understood. Using a variety of approaches, we now identify a conserved intronic element that apparently plays important roles in regulating the cell-specific expression of SF-1.

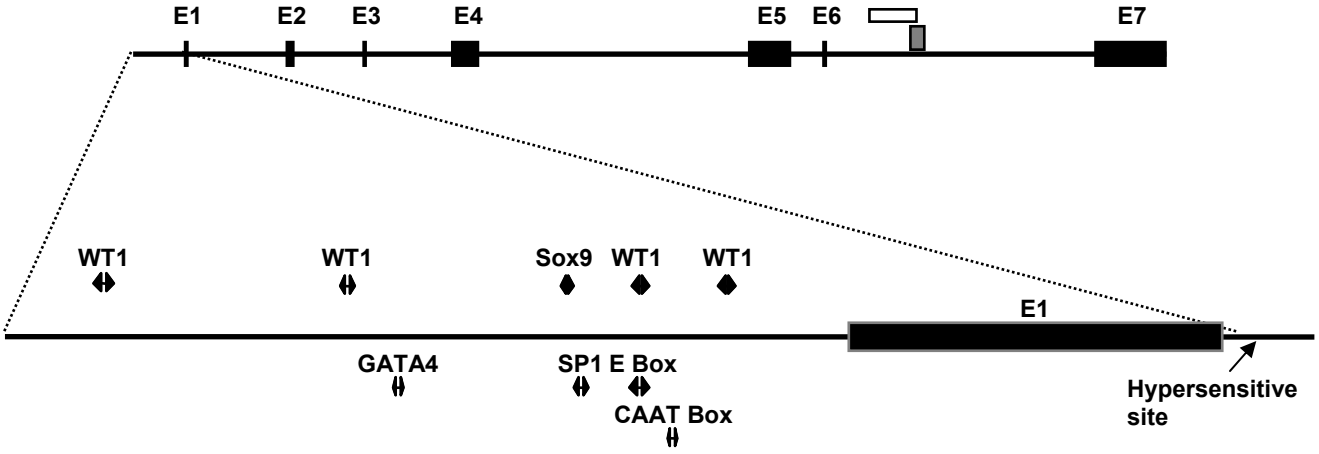


Figure 6-1 Schematic diagram of the *Nr5a1* locus.
Shown is the structural organization of the mouse *Nr5a1* gene. The expanded region shows elements in the proximal promoter that have been implicated in SF-1expression. Also shown is the DNase I probe (white box) and conserved region (grey box) within the 6th intron whose function is described below.

6.2 Results

DNase I digestion reveals a tissue-specific DNase I hypersensitive site in the 6th intron of the *Nr5a1* locus

To identify potential regulatory elements, we adopted a more global analysis of the *Nr5a1* locus. A well-established method of identifying regulatory elements is to define chromosomal regions in the nucleus that are hypersensitive to DNase I digestion; this hypersensitivity reflects an open chromatin state that often is associated with actively-transcribed genes. As a control, a previously described hypersensitive site in the first intron of the Nr5a1 locus (Figure 6-1) was used to confirm that the assay was working (Ninomiya et al., 1996; Nomura et al., 1996). Once we were able to replicate the results (Figure 6-2), we began to screen for novel sites using various *Hind*III fragments from the mouse Nr5a1 locus. As shown in Figure 6-3, one probe of 1858 bp revealed a strong hypersensitive site within the last intron of the Nr5a1 gene. We next investigated if this DNase I hypersensitivity was seen in other cell lines. Interestingly, this interaction was seen in Y1 adrenocortical and MA-10 Leydig cells, but not in 3T3 fibroblasts or αT3 gonadotropes, suggesting that it is associated with steroidogenic cell lines (Figure 6-4).

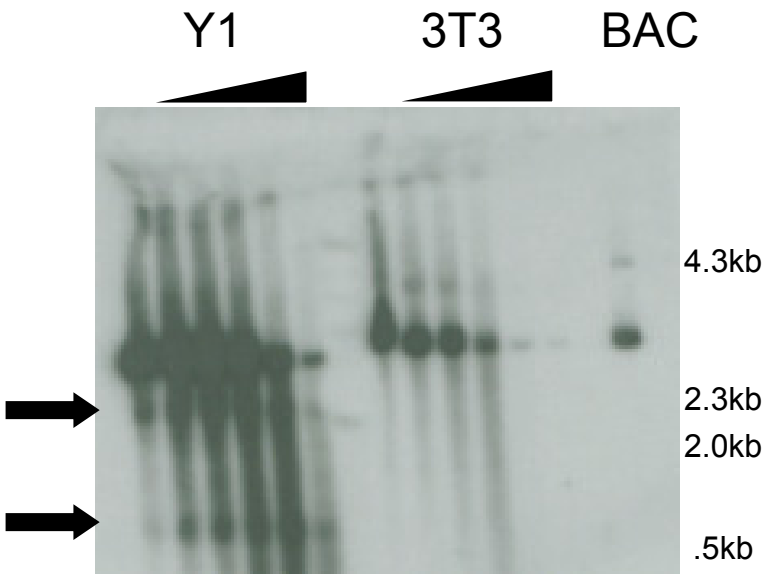


Figure 6-2 Hypersensitive site in SF-1 Intron 1
Nuclei were isolated from Y1 and 3T3 cells and DNAse I digestion and mapping was performed as described in Chapter 2. Increasing DNase I concentrations are indicated by the wedge. The band from the BAC was used as a control for the probe. The arrows indicate DNAse I hypersensitive sites that were detected with a 3kb *HindIII* fragment located in the 1st intron of SF-1. This was a previously described site and was used as a control.

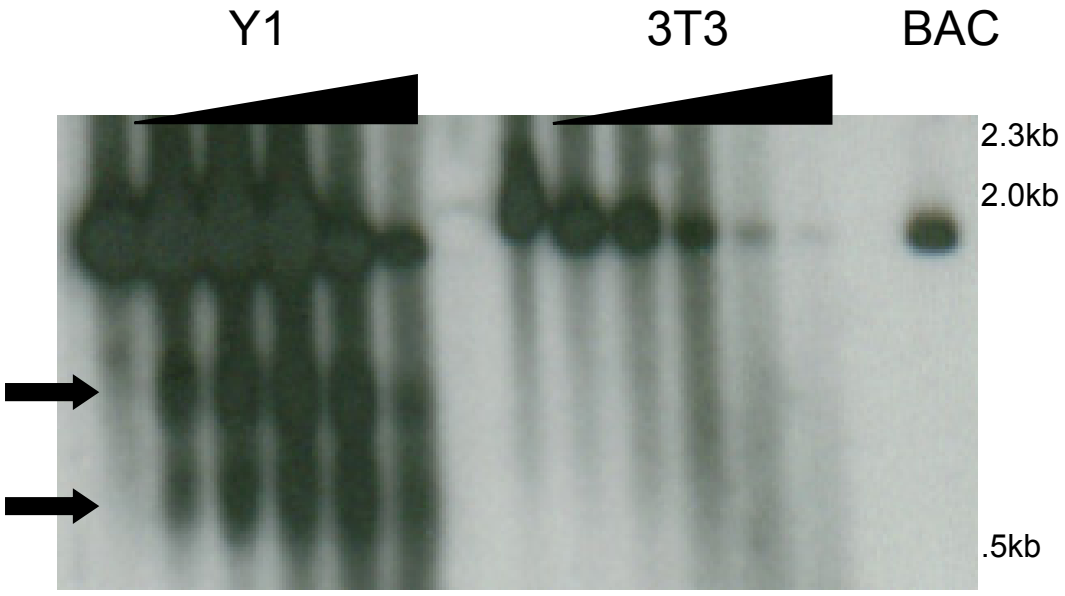


Figure 6-3 DNAse I hypersensitivity mapping of the *Nr5a1* locus in Y1 Cells
Nuclei were isolated from Y1 and 3T3 cells and DNAse I digestion and mapping were performed as described in Chapter 2. Increasing DNase I concentrations are indicated by the wedge. The arrows indicate DNAse I hypersensitive sites that were detected with a 1858 bp *HindIII* fragment located in the 6th intron of SF-1. The hypersensitive site is detected in Y1, but not 3T3 cells. The band from the BAC was used as a control for the probe.

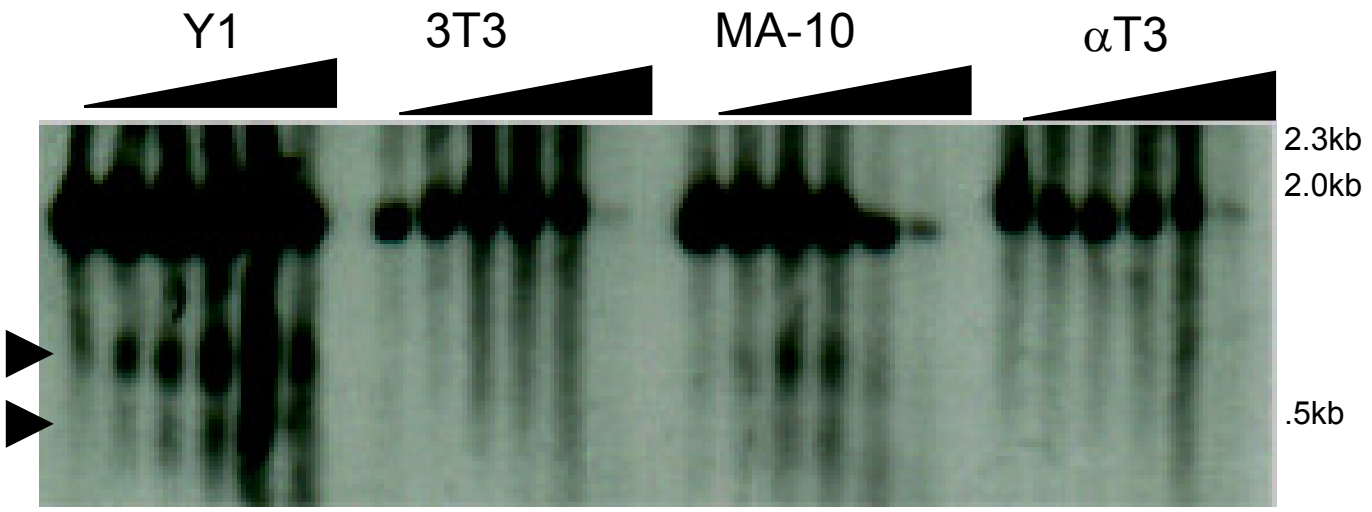


Figure 6-4 DNAse I hypersensitivity mapping of the *Nr5a1* locus in Y1 and MA-10 Cells
Nuclei were isolated from the indicated cell lines and DNAse I digestion and mapping was performed as described in Chapter 2. Increasing DNase I concentrations are indicated by the wedge. The arrowheads indicate DNAse I hypersensitive sites that were detected in Y1 and MA-10 cells with a 1858 bp *HindIII* fragment located in the 6th intron of SF-1.

To gain further insights into potential roles of this tissue-specific DNase hypersensitive site, we compared the sequence of the hypersensitive site probe to sequence from human and chicken. As shown in Figure 6-5, these sequence comparisons revealed a 452bp region that was highly conserved between mouse and human SF-1. This conservation was 87% across the 452 bp region with identity of 401/452 bp. When the comparison was extended to chicken DNA the identity was 208/452 for 45% identity. A smaller ~135 bp segment was even more highly conserved from mouse to chicken, with an identity of 101/135 bp, or 74%. As such conserved intronic sequences often denote important regulatory elements (Levy et al., 2001; Wasserman et al., 2000), this high degree of sequence conservation suggests that this conserved intronic region may play important roles in SF-1 regulation.

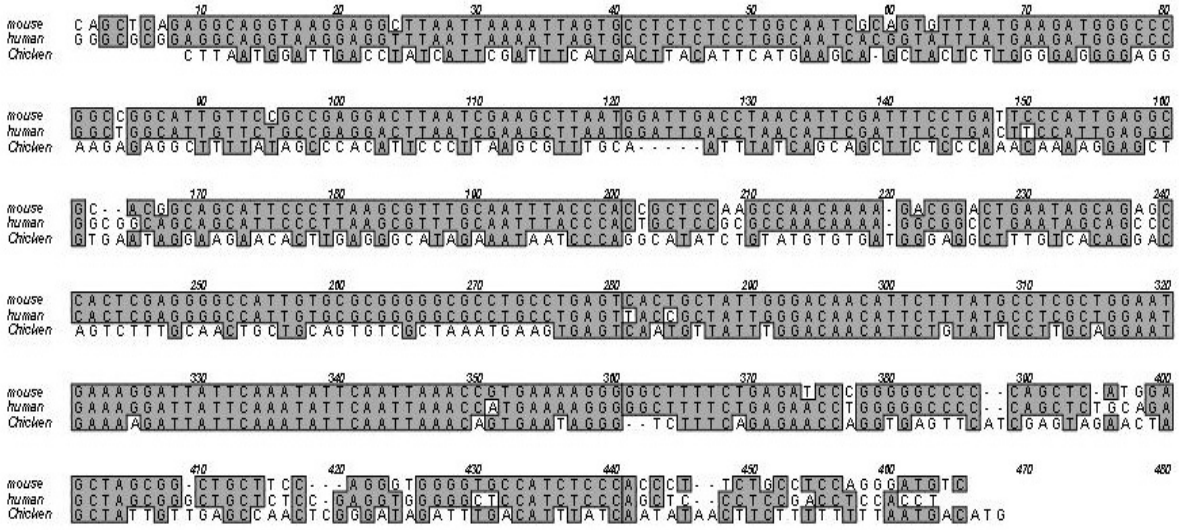


Figure 6-5 Sequence of the conserved intronic region.
Shown are the sequences of the corresponding region of the *Nr5a1* genes from mouse, human, and chicken genomes. Note that there is a 452 bp region that is conserved between mouse and human, and a 135 bp region that also is conserved in the chicken sequence.

Comparison of Genomic Locus containing mouse and human NR5A1

To gain a more global comparison of the Nr5a1 locus, mouse, rat and chicken genomic sequence was aligned to human sequence using VISTA genomic alignment software (Frazer et al., 2004). As shown in Figure 6-6, the exons of SF-1 are conserved with high sequence identity in all four species. The proximal promoter region of human, mouse and rat also have high sequence identity. The human, mouse, and rat genes have several regions of conservation in intronic regions that may also be potential regulators of SF-1 expression. Including the chicken gene in this analysis, however, limits the area of close conservation (outside of the exons) to the 6th intron element studied here. Such a global gene analysis with multiple species, which has only recently been possible, can provide important insight into genomic regulatory regions (Frazer et

al., 2004). The sequence alignments, in conjunction with the DNase I results, strongly suggest that intron 6 merits more study.

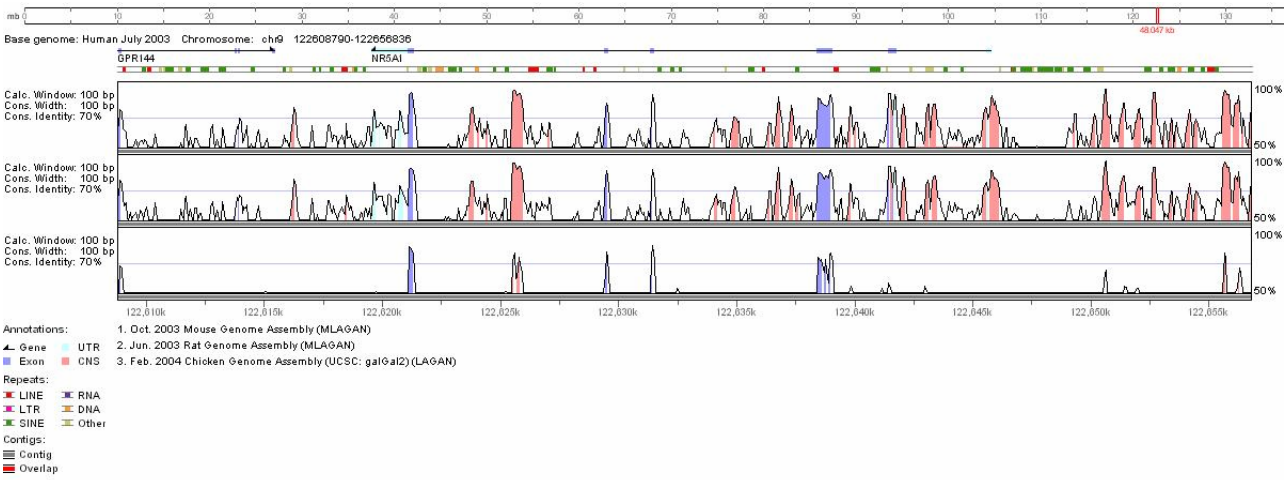


Figure 6-6 Comparison of genomic sequences for 50kb around the Nr5a1 locus reveal other sites of DNA conservation.
Mouse, rat and chicken genomic sequence is compared to human sequence. SF-1 is in the reverse orientation on chromosome 9 and in this schematic. Exons are indicated above the alignment by blue boxes. In the histogram, conserved exon sequence are noted by light blue color and conserved non coding sequence by pink. The top histogram is mouse sequence compared to human, the middle is rat compared to human, and the bottom is chicken compared to human. Note the conserved region in intron 6.

The conserved intronic region stimulates SF-1 promoter activity in a cell-specific manner.

To examine the potential role of the conserved intronic sequence in *SF-1* gene regulation, we performed transient transfection experiments in which different parts of entire region were placed upstream of a construct in which 850 bp of the 5'-flanking region of SF-1 drives luciferase expression (p850SF1-luc). This strategy was adopted to avoid potential confounding influences if both positive and negative regulatory elements are found in the region. The p850SF1-luc construct was first tested for activity in Y1, MA-10, αT3, and 3T3 cells.

Similar to a previous report (Harris and Mellon, 1998), the proximal 5'-flanking region of SF-1 in p850SF1-luc directed the highest levels of luciferase activity in Y1 adrenocortical cells, with intermediate activity in MA-10 Leydig cells, lower levels in αT3 cells, and no activity in 3T3 cells (Figure 6-7).

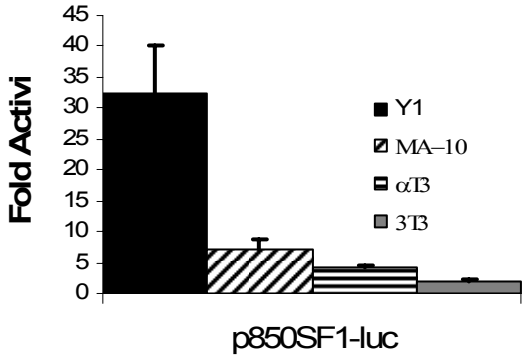


Figure 6-7 Activity of p850SF1-luc in Y1, MA-10, αT3, and 3T3 cells
Fold activity was calculated in each cell line using empty vector as the normalizer. Y1, MA-10, αT3, and 3T3 cells were transfected using the calcium phosphate method described in Chapter 2.

As shown in Figure 6-8, several subregions of the conserved sequence independently stimulated activity of the proximal SF-1 promoter, but with different patterns of tissue-specificity. For example, the 1:72 bp fragment increased promoter activity in Y1, MA-10 and αT3, whereas other fragments (e.g., 225:359 and 314:452) led to significant increases in promoter activity only in the steroidogenic Y1 adrenocortical and MA-10 Leydig cells. These findings support a functional role of this conserved intronic element in the tissue-specific regulation of SF-1. An interesting observation is that all of the subregions independently stimulated promoter activity in Y1 and MA-10 cells, while a modest effect, if any, was seen with the entire1:452 region (Figure 6-8). This may reflect

the presence of negative regulatory elements that are disrupted by the preparation of the specific subclones examined.

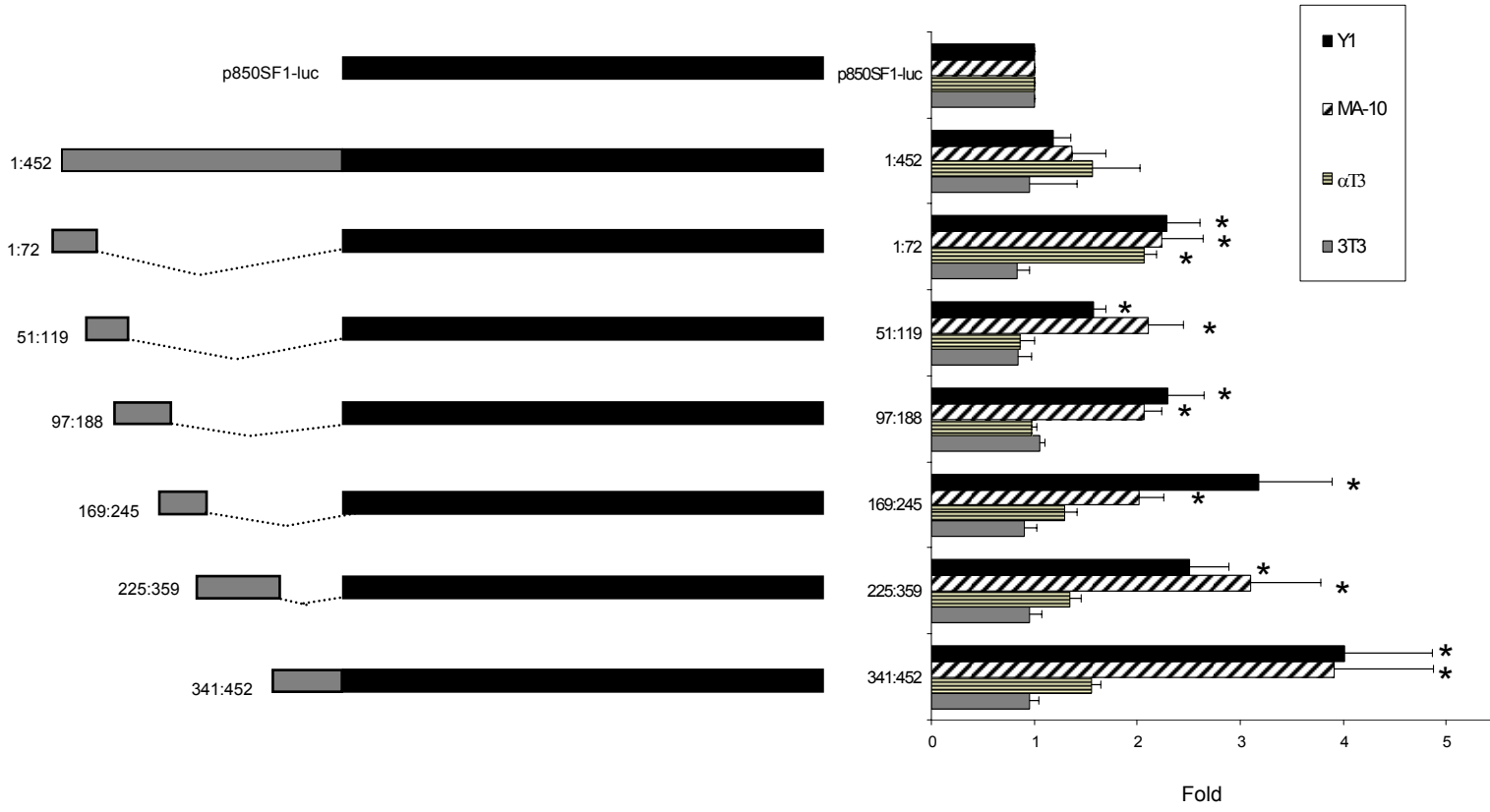


Figure 6-8 The conserved region stimulates promoter activity of the proximal 5'flanking region of SF-1 in a cell-specific manner.
Y1, MA-10, αT3 and 3T3 cell lines were transiently transfected with a reporter construct containing all or part of the conserved intronic sequence upstream of p850SF1-luc as described in Chapter 2.
Results are presented normalized for each cell line with the p850SF1-luc as 1 fold and error bars are +/- standard error of means. *=p<.05.

The homeodomain transcription factors Pbx1 and Meis1 positively regulate SF-1 expression via the conserved intronic element.

A computer query of the conserved intronic region for motifs matching binding requirements of known transcription factors predicted a number of potential binding sites for known transcription factors, many of which were AT-

rich sequences that correspond to potential binding sites for homeodomain proteins. Several of these binding sites are diagramed in Figure 6-9.

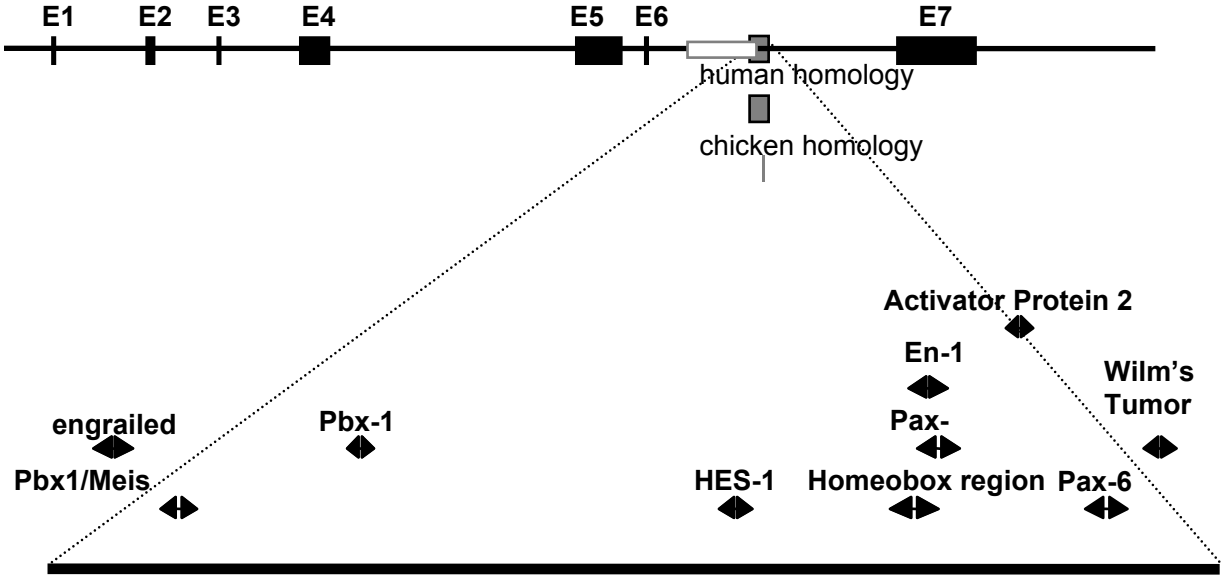


Figure 6-9 Schematic diagram of the *Nr5a1* locus with location of DNase I probe and conserved intronic sequence.
White box indicates DNase I and grey boxes are conserved sequences.
The expanded region shows selected transcription factor binding sites predicted by MatInspector.

In particular, one sequence (CTCCTGGCAATCGCAGT) at 46:62 matched the cooperative binding site of the homeodomain transcription factors Pbx1 and Meis1. This finding is of considerable interest because KO mice lacking Pbx1 have adrenal agenesis and gonadal defects (Schnabel et al., 2003), and previous studies showed that both Pbx1 and Meis1 are expressed by mouse Y1 adrenocortical tumor cells, where they stimulated promoter activity of the 5'-flanking region of 17α-hydroxylase via the CRS1 element (Bischof et al., 1998). Pbx1 is also expressed in the LβT2 cell line where it is involved in the regulation of the FSHβ-subunit gene (Bailey et al., 2004) and in the GT1-7 GnRH neuronal cell line where it is able to regulate the expression of the GnRH gene (Rave-

Harel et al., 2004). As shown in Figure 6-10A, Y1 nuclear extracts bound the Pbx1-Meis1 probe to form a shifted complex. This complex was specifically affected by an anti-carboxyl terminal Pbx1 antiserum, demonstrating that the complex includes Pbx1. In contrast, two antisera against Meis did not affect the complex. While this may reflect limitations of the antisera, it is possible that Meis is not contained in this complex. A similar complex was also formed by the steroidogenic MA-10 cells and αT3 pituitary gonadotropes, suggesting that Pbx1 is also expressed in MA-10 cells and αT3 cells (Figure 6-10B).

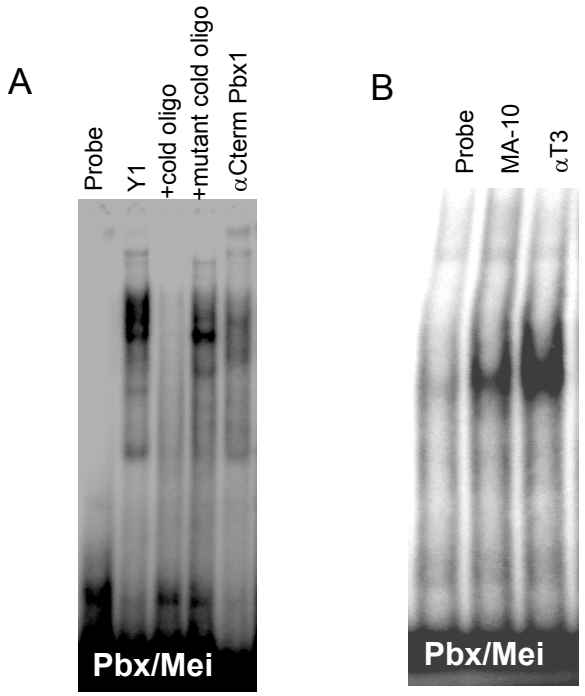


Figure 6-10 Proteins present in Nuclear extracts from Y1, MA-10 and αT3 cells bind to DNA containing the compound Pbx/Meis binding site
A. A complex is formed in Y1 cells on the Pbx/Meis oligo. An C terminal antibody to Pbx disrupts this complex.
B. A complex is formed in MA-10 and αT3 cells on the Pbx/Meis oligo.

We next examined the role of the putative Pbx1-Meis1 binding site in the transcriptional activity of the conserved motif. Altering the compound Pbx1-

Meis1 motif by site-directed mutagenesis decreased the activity of 1:452 by 20% in Y1 and MA-10 cells and 40% in αT3 cells (Figure 6-11). These findings suggested that Pbx and Meis might regulate SF-1 gene regulation via effects on this conserved sequence. To demonstrate directly that Pbx1 and Meis1 interacted with this region, we performed gel mobility shift assays with a probe comprising the element and nuclear extracts from different cultured cell lines.

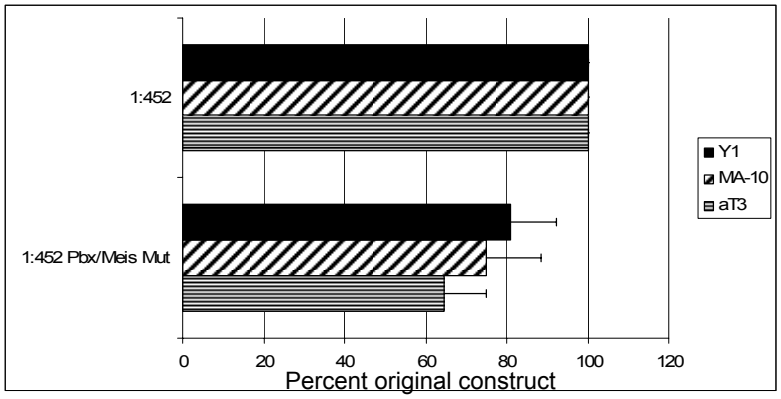


Figure 6-11 Mutation of the Pbx/Meis binding sites reduces enhancer activity of the intronic region.
Site directed mutagenesis was used to introduce mutations into the Pbx/Meis binding site. Mutations were introduced in the full length 1:452 conserved region through site directed mutagenesis. Results are presented normalized with the non-mutated construct as 100% and error bars are +/- standard error of means. *=p<.05

6.3 Discussion

Although analysis of this conserved intronic element is incomplete, the present data suggest that this element is an important regulator of the tissue-specific expression of SF-1. Detailed investigation of other potential binding sites is still needed, as well as *in vivo* data. However the current data, coupled with the PBX1 KO mice, suggests that Pbx is a regulator of SF-1 expression. Intronic regulatory elements, although not as well studies as promoter regions, are

becoming more commonly characterized. Other genes with potential intronic enhancer regions identified through DNase hypersensitivity and sequence analysis include adenosine receptor 2B (Braas et al., 2003), Ndrg4 (Maeda et al., 2004), Pax6 (Kleinjan et al., 2004), glutamine synthetase (Garcia de Veas Lovillo et al., 2003), dopamine transporter gene (Greenwood and Kelsoe, 2003), colony stimulating factor receptor (Follows et al., 2003), AP-2 (Zhang and Williams, 2003), TNF-α (Barthel and Goldfeld, 2003), NAC1 (Mackler et al., 2003), BCL3 (Ge et al., 2003), and FGF8 (Gemel et al., 1999). This number is likely to increase as a growing number of computer programs are available to predict conserved noncoding regions as potential regulators of gene expression (Fedorova and Fedorov, 2003; Frazer et al., 2004; Hare and Palumbi, 2003; Levy et al., 2001; Majewski and Ott, 2002; Xue et al., 2004).

Although the mechanisms remain undefined, previous studies have demonstrated links between homeodomain proteins and SF-1 gene regulation. For example, KO mouse studies have implicated both Lhx9 and Arx as important upstream regulators of SF-1 within the gonads. Lhx9 KO mice had gonadal agenesis associated with markedly decreased SF-1 expression and impaired proliferation (Birk et al., 2000). Similarly, Arx KO mice had gonadal dysgenesis (Kitamura et al., 2002). In contrast, Pbx1 KO mice also had impaired gonadal development, with diminished SF-1 expression in the interstitial region, but—more strikingly—had complete adrenal agenesis (Schnabel et al., 2003). The emerging picture thus suggests that distinct homeodomain and HOX proteins differentially regulate the expression of SF-1 in the adrenal cortex and gonads,

and most likely the pituitary and hypothalamus. Based upon expression patterns, other homeodomain candidates for this regulation include Emx2 (Miyamoto et al., 1997), TOX (Kang et al., 2004), and Lbx2 (Chen et al., 1999).

Pbx1 and Meis 1 are both expressed in multiple tissues, and it perhaps is surprising that they should regulate the tissue-specific expression of genes like SF-1. However, it is apparent that these genes can interact cooperatively with other genes of the homeotic complex to regulate the expression of key developmental genes. Previous studies in Y1 adrenocortical cells showed that both Pbx1 and Meis1 were expressed in these cells, where they regulated the expression of steroid 17α-hydroxylase via the CRS1 promoter element (Bischof et al., 1998). This finding was somewhat surprising, because the region of the CYP17 promoter that bound Pbx1-Meis1 was not conserved in other species, and because similar elements were not found in genes encoding other cytochrome P450 steroid hydroxylases. Our findings raise the possibility that one component of the observed effect of Pbx1-Meis1 cotransfection may reflect the ability of these proteins to stimulate the expression of SF-1, which is known to regulate CYP17 promoter activity.

It is instructive to relate our findings with Pbx1-Meis1 with recent results from Pbx1 KO mice. Y1 adrenocortical and MA-10 Leydig cells behaved identically in experiments shown here, suggesting that Pbx1 and Meis1 play similar roles in the steroidogenic cells of the adrenal cortex and gonads. In contrast, although Pbx1 KO mice had adrenal agenesis, their testes were still present, suggesting that the roles of Pbx1 differ in adrenal glands and gonads.

Of note, although their testes were present, Pbx1 KO mice exhibited impaired SF-1 expression in the interstitial region of the testes where the Leydig cells reside. Thus, we envisage that Pbx1 likely regulates SF-1 gene expression in both adrenocortical progenitors and in Leydig cells. In contrast, we propose that other transcription factors likely regulate SF-1 expression in Sertoli cells. Another unresolved question is whether Pbx1 and Meis1 regulate SF-1 expression in the ovary. A recent report found expression of Pbx1, Pbx2 and Meis2, and two proteins related to Meis, PREP-1 and PREP-2, in the mouse ovary (Villaescusa et al., 2004). Adrenocortical and Leydig cells each comprise the sole steroidogenic cell in their respective tissues. In contrast, ovarian steroidogenesis requires cooperative interactions between theca cells, which express most of the steroidogenic enzymes, and granulosa cells, which express aromatase and carry out the terminal reaction in estrogen biosynthesis.

The complex cell specific and developmental expression of SF-1 most likely reflects a combinatorial process involving multiple transcription factors. A comparison of Hox protein expression in testis and adrenal glands show that many of the same Hox genes are expressed in both tissues, although at different levels (Takahashi et al., 2004). It will be very interesting to see which, if any, of these homeobox proteins are involved in the regulation of SF-1 expression in these tissues. It also will be very important in future studies to examine the effect of this conserved intronic element in transgenic mice, to see if its functions in vivo are linked to steroidogenic cells or possibly also extend to other sites such as the VMH.

Chapter 7 Conclusions and Future Directions

7.1 Creation of eSF-1/eGFP linage marker

Discussion of Results/Why these results are important

The 50kb SF-1/eGFP transgene provided a novel tool to study the fate of cells in the SF-1 KO mice. The reporter transgene allowed us to study the fate of these cells in the KO mice in a manner not previously possible for SF-1. The 50kb SF-1/eGFP transgene also is a useful tool to sort SF-1 expressing cells from neighboring cells, particularly with heterogeneous populations of cells such as developing hypothalamus and gonads. The transgene did not completely recapitulate endogenous expression of SF-1. Disparities were seen in the postnatal Sertoli cells of the testis, pituitary gonadotropes, and the developing spinal cord. SF-1 expression is normally seen in Sertoli cells and pituitary gonadotropes, but eGFP expression was not observed.

SF-1 expression has not been described in the developing spinal cord, although another SF-1 reporter transgene expresses in the spinal cord (Wilhelm and Englert, 2002) and a Dax-1 reporter transgene is also expressed in this region (Swain et al., 1998). Recent data from our laboratory suggests that a Cre-transgene made from the same 50kb SF-1 fragment also expresses in the developing spinal cord (N. Bingham, personal communication). Previous *in situ* and immunohistochemical examination of embryos using a SF-1 probe or specific

antibody has not shown any SF-1 expression. The possibilities are that this is ectopic expression or a novel site of expression that is below the levels of expression that can be detected by in situ or immunohistochemistry. The data from the other transgenic lines suggests that it could be a novel site of SF-1 expression, especially since a Dax-1 transgene also expresses in this region. SF-1 and Dax-1 are co-expressed in many sites, suggesting that they are controlled by similar mechanisms. Therefore, it is possible that both of these proteins are expressed in this region. It is also possible that regulatory regions that normally suppress the expression of SF-1 and Dax-1 in the spinal cord are missing from the various transgenic constructs. Ongoing examination of expression data in more transgenic lines expressing Cre will lend some insight into this issue.

Where will we go next

Although we have not determined whether the differences in expression are due to the integration site of the transgene or the lack of regulatory elements in the transgenic construct used, further studies could be used to determine which of these hypotheses are correct. Several lines of experiments could be used to investigate what genomic sequences are necessary for SF-1 expression in different cell types. First, it would be important to analyze several other lines made from the same eGFP construct. This would allow for identification of any expression results that were due to the transgene integration site. Multiple transgenic constructs encompassing additional genomic sequences could be used to explore whether more 5' or more 3' genomic sequences are needed to activate expression of the reporter in Sertoli cells

and gonadotropes and/or silence expression in the spinal cord. Once such experiments have provided results using large DNA segments, deletion constructs could be used to narrow the region where important regulatory domains are present. Individual regulatory elements could be further defined using mutations to these transgene constructs to show that identified regulatory elements are necessary for transgene expression in certain tissue/cell types.

Importantly, this construct and this line of mice will allow for this transgene to be used as a tool to track and study the development of SF-1 containing tissues. The transgene will also allow for the separation of SF-1 expressing cells from the surrounding cellular milieu. This technique has been successfully used to sort eGFP positive cells from negative cells. These experiments are discussed below. In addition several collaborative studies have been undertaken that use the SF-1/eGFP reporter transgene. One collaboration with Stu Tobet at Colorado State University uses the transgene to study the migration of neurons in the developing VMH using real time video microscopy. Dr. Tobet has used this approach to study the migration of GnRH neurons into the hypothalamus. Dr. Tobet's is using the real time imaging of the cells migrating using cultured hypothalamic slices to study the effects of the SF-1 knockout on cell migration. These studies are still in a preliminary stage, but the SF-1/eGFP transgene is proving to be a useful tool in these studies.

Another collaboration using the SF-1/eGFP transgene is with Serge Nef at the University of Geneva. Dr. Nef's group has used the transgene to identify differentially expressed genes in the developing gonads of female and male mice at

different developmental time points. Dr. Nef used FACS to sort the eGFP positive cells of the developing urogenital ridge from female and male embryos and then used Affymetrix gene chips to investigate the developmental profile of genes. Dr. Nef showed that markers that have been previously described in these cells, such as MIS (Mullerian inhibiting substance) and SCC (side chain cleavage) in the male and wnt4 and follistatin in the female, are present and differentially regulated as expected. They also identified many novel female specific genes. These studies will yield many important insights into the early stages of gonadal development.

7.2 Identification of novel SF-1 target genes

What we learned/Why these results are important

The SF-1/eGFP transgene was used to separate eGFP expressing cells of the developing hypothalamus from non-eGFP positive cells. The reporter transgene allowed us to sort fluorescent cells from both SF-1 WT and SF-1 KO embryos at E16.5. Cells were then used for microarray analysis and QRT-PCR to identify genes with reduced expression in the SF-1 KO. This method allowed us to identify potential novel SF-1 target genes in the VMH.

Initial experiments were performed to determine the viability of using the FACS approach to sort cells in the developing hypothalamus. Immunohistochemistry was used to determine that the eGFP positive cells were still present in KO mice, but their distribution was altered to that they no longer condensed to form the VMH. Cell counts were performed on both the

immunoreactive cells and cells sorted from the VMH by FACS to show that there was no alteration in eGFP cell number in the KO mice compared to the WT mice. The immunohistochemistry results suggested that the failure of the VMH to form properly in the KO mice was due to a defect in migration of these cells to their normal position in the brain.

After several preliminary experiments showed that it was possible to sort the eGFP positive cells, FACS was used to collect cells to use for Affymetrix arrays. Many interesting genes were identified in the screen for novel target genes. Since available data suggested that the KO had a defect in cell migration, genes (or gene families) previously identified as important in cell migration were focused upon. Several of these genes will warrant further investigation to determine if they are direct target genes of SF-1 or if the reduced expression of the gene is secondary to developmental effects of lacking SF-1. A parallel screen and analysis done by collaborators at Rockefeller University corroborated our results by the identification of two of the genes in the independent screens. Although the use of different arrays limited the ability to directly compare all of the genes identified, the collective data argue strongly that cerebellin 1 and ten-M2/odz2 are exciting candidates as SF-1 target genes.

Next quantitative real time PCR was used to confirm which of the identified genes were in fact down-regulated in the KO embryos. Cbln1, ten-M2/odz2, Eps15, Dab2, and Ten-C were genes with reduced gene expression confirmed by QRT-

PCR. Cbln1 also was shown to have reduced expression in the VMH of SF-1 central nervous system specific KO mice.

Future Directions

The QRT-PCR was very difficult to perform due to the limited amounts of RNA that could be obtained and the large number of genes to be evaluated. Due to these limitations, a small number of the genes that were down-regulated from the arrays were chosen to be analyzed using QRT-PCR. Analyzing a small number of genes left many more interesting genes to evaluate. One recently developed commercial product has become available that could help with this problem. A product from NuGEN (Ovation System RNA Amplification) uses as little as 5ng of RNA to produce microgram amounts of cDNA for real time PCR. In addition to validating more genes by QRT-PCR, it would be useful to do additional arrays to identify more genes with downregulated expression.

Further study of these differentially expressed genes will be necessary to determine if they are direct targets of SF-1 or if their expression is reduced secondary to other indirect mechanisms. The developmental profile of gene expression in the VMH needs to be determined for the majority of the hits, as the identified VMH expression is a novel site of expression for many of the genes. A careful developmental expression profile in both wild type and SF-1 knockout genes should be undertaken. Several of the genes have well characterized *in situ* probes or specific antibodies that can be employed in these studies. There are well characterized antibodies to ten-M2 (Zhou et al., 2003), ten-c (Chemicon), and

EPS15 (Offenhauser et al., 2000). *In situ* probes to Foxg1 (Hanashima et al., 2002) and Dab2 (Morrisey et al., 2000) are also available.

The use of several of the developed knockout models for several of these genes could be used to explore the expression of SF-1 as well as other genes in these mice. The use of these mice for expression studies will assist in the identification of pathways that are present in the developing VMH and help identify some of the expression alterations that are secondary to the loss of SF-1 expression. Mouse knockout lines available are Dab2 (Morris et al., 2002), BDNF conditional (Rios et al., 2001) and BDNF global (Kernie et al., 2000), Tbr1 (Hevner et al., 2002), ten-c (Mackie and Tucker, 1999), and Foxg1 (Pratt et al., 2004).

Another line of studies to identify direct targets of SF-1 could employ promoter analysis of the genes. Using computer prediction programs, the promoters of the genes could be screened for the presence of SF-1 binding sites. Identified sites could be further investigated with EMSA, transient transfections using reporter genes, and chromatin immunoprecipitation (CHIP). While EMSA and transient transfections are techniques that have been well described for identifying SF-1 target genes, less success has been obtained for chromatin immunoprecipitation. A recent paper has had success with the technique, showing SF-1 bound to the promoter of one of its target genes, steroidogenic acute regulatory protein (Hiroi et al., 2004). The antibody used in this study is difficult to obtain, but we have recently developed an antibody produced against the ligand binding domain of SF-1 that might work for CHIP.

The success of this screen, along with the parallel development of technology to support this technique, suggest that this method could be used to screen for target genes of SF-1 in other target tissues such as the developing gonads and adrenal glands where the known target genes in these tissues provide no clues as to why these tissues do not develop. In addition the data could be resorted and analyzed by sex of the embryo pools to identify any genes that are differentially expressed by sex of the embryo.

7.3 BDNF Promoter Analysis

What we learned

One potential target gene of SF-1 that was examined in more detail was brain derived neurotropic factor (BDNF). BDNF was chosen because there was preliminary evidence independent of the array results that suggested that BDNF could be a direct target of SF-1. It had been shown that BDNF expression is decreased in the VMH of SF-1 KO mice (Tran et al., 2003) and current work in the laboratory had shown BDNF expression to be reduced in the VMH of SF-1 CNS-KO mice. In addition there is also a striking similarity between the phenotype that is seen in the SF-1 adrenal rescued mice and the BDNF heterozygous mice. Both of these mice develop a late onset obesity. For these reasons we decided to look at BDNF as a target gene of SF-1.

Analysis of the BDNF genomic locus revealed that promoter 4 of BDNF had three candidate SF-1 binding sites, which bound SF-1 in EMSA. Luciferase reporter

constructs were made to assess the ability of SF-1 to enhance transcription of these reporter constructs. Results of these experiments showed that the co-transfection of SF-1 increased expression of the reporter construct by 1.54 fold. Similar results were seen in two cell lines, COS and PC-12. Mutation of the binding sites eliminated any statistically significant increase in promoter activity. Binding assays revealed that SF-1 proteins were able to bind to all three of the predicted binding sites. Strongest binding was seen with the elements at -651 bp and -244 bp. The element at -144 bp manifested the weakest binding. *In situ* hybridization of the VMH showed greatly reduced expression levels in the CNS-specific SF-1 KO mice, while expression of BDNF in the surrounding brain regions remained unchanged.

Future Directions

While the above results are not clear cut in determining whether BDNF is a bona fide target gene of SF-1, they are suggestive that this is the case. The transfection results are limited due to the available cell lines in which to do the transfections. There is not a hypothalamic cell line that expresses SF-1 and the cell lines used to do these experiments do not express SF-1. PC-12 cells, although often used to study BDNF, are not of neuronal origin. They are pheochromocytoma cells, derived from adrenal medullary tumors. One proposed set of experiments to overcome this lack of a proper cell line is to create one using the SF-1/eGFP mice. The ability to sort the SF-1 expressing cells from the surrounding cells using the eGFP transgene will allow us to create a cell line using the eGFP marker coupled with a T-antigen “Immortal Mouse” (Jat et al., 1991). The T-antigen mouse can be

bred to the eGFP transgenic line and embryonic neuronal preparations made. By using cell preparations that are sorted for eGFP expression and the T-antigen transgene, we can immortalize the cells and monitor the ability to continue to express the SF-1/eGFP transgene. Although numerous technical problems could arise, we feel this is an idea that is worth trying to see if we can get to work. Mice are currently being bred for this purpose.

Additionally to assess in a quantitative manner the expression of BDNF in the SF-1 KO mice, we are working on QRT-PCR in FACS sorted eGFP cells. Early attempts with E16.5 cells were unable to amplify enough product to properly evaluate the results. Since BDNF expression increases dramatically after E16.5 (Marmigere et al., 1998; Tran et al., 2003), we used E18.5 embryos as a source for RNA. Initial experiments at this time point, show a reduction in all transcripts of BDNF by 50% in the KO embryos (L. Zhao, personal communication).

7.4 Novel SF-1 regulatory element

What we learned/Why these results are important

A screen for novel regulatory elements of SF-1 used DNase I hypersensitivity mapping to look for regions of open chromatin. The rational behind this screen was that open regions of chromatin often correspond to regulatory regions (Crawford et al., 2004). The proximal promoter of SF-1 has been well characterized, but has not revealed any regions that could account for the complex cell and tissue specific

manner in which SF-1 is expressed. For this reason we chose to used DNase I hypersensitive mapping to screen the genomic locus to look for regions of open chromatin and use this region as a starting place to explore their ability to act as regulatory regions of SF-1 expression.

Although the screen was not exhaustive of the entire genomic locus, one region of open chromatin was identified in the screen. The identified region mapped to the SF-1 intron between exon 6 and exon 7. A local sequence alignment revealed high sequence identity between mouse and human intronic sequence, and a smaller conserved sequence in the chicken intronic sequence. In addition when the entire genomic region was compared between mouse, rat, human, and chicken sequence, the identified hypersensitive site was easily identified as a conserved non coding region between all four species.

Transient transfections revealed that various subfragments of this region were able to act as an enhancer of SF-1 promoter activity. Computer analysis used to predict binding sites showed numerous binding sites in the region. One of the predicted sites was a compound Pbx/Meis binding site. This site interesting because a recently published paper detailed adrenal and gonadal defects in the Pbx1 KO mice (Schnabel et al., 2003). The Pbx KO mice lacked adrenal glands and the gonads did not develop correctly. In addition the expression of SF-1 was reduced to minimal levels in the Pbx KO mice. Analysis of this binding site showed that nuclear extracts from Y1, MA-10 and αT3 cells had proteins that were able to bind to this sequence. Site-directed mutagenesis of this site revealed that mutation

of this site eliminated its ability to act as an enhancer in transient transfection analysis.

Another caveat of these experiments is was our inability to assess the intronic region as a regulatory element important in the expression of SF-1 in the VMH. No cultured cell lines exist that are a good model for SF-1 expression in the VMH, so analysis of this conserved intronic region in the regulation of SF-1 expression in the VMH will have to be conducted *in vivo* through the use of transgenic constructs.

Future Directions

Future experiments will further examine the conserved region, especially those subregions with the greatest effect of promoter activity. Other regions of close examination will include the subfragment that has close conservation with the chicken intron. Another predicted binding site that was of interest was an engrailed binding site at 21:37, AGGCTTAATTAAAATTA. This was interesting because intronic engrailed and Pbx/Meis sites have been shown to work in conjunction in the regulation of Fgf8 (Gemel et al., 1999). We were unable to show any binding to the engrailed site, most likely because engrailed is expressed in neuronal cells and none of the nuclear extracts that were analyzed were of neuronal origin. Preliminary data shows that binding can be seen with Y1, MA-10 and αT3 nuclear extracts and a probe that encompasses a predicted PAX binding site at bp 342.

One way of identifying proteins that are binding to probes used for the gel shifts is to use modified affinity chromatography coupled with mass spectrometry to identify the proteins that are bound to these probes. This can be accomplished by

the use of biotin labeled oligos coupled to streptavidin labeled beads to pull proteins that bind to the oligos out of the nuclear extract. These proteins can then be identified by mass spectrometry (Drewett et al., 2001). This approach could be a very important tool to identifying the specific proteins bound to the probes, as the probes can bind several protein complexes and multiple different proteins. For example, Pbx is very often found in complex with homeodomain proteins and this technique could be very useful in resolving the binding partners of Pbx in our cell lines.

The SF-1 genomic locus analysis revealed other regions of conservation between mouse and human. A similar approach could be used to investigate these regions for ability of act as regulatory regions. As seen in Figure 6-4, several other regions in the mouse, human, and chicken genomic regions had high conservation. Therefore these regions could contain additional regulatory regions. It is possible that these additionally intronic conserved regions have distinct roles in the tissue and cell specific expression of SF-1

Thus, the body of work described in this dissertation reports several important advances in the study of SF-1. First, the SF-1/eGFP transgenic mouse represents the first SF-1 BAC transgenic mouse and to date, the best reporter of endogenous SF-1 expression. Second, using the SF-1/eGFP transgenic mouse, we identified putative target genes of SF-1 in the ventromedial hypothalamus. These target genes will help characterize the role of SF-1 in the VMH. Third, we identified a novel intronic regulatory region of SF-1 using DNase I hypersensitive mapping which

appears to have a role in the tissue specific expression of SF-1. Together, these findings represent a significant body of work and will be used as the foundation of many future research projects.

Chapter 8 References

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APPENDIX 1 SF-1 TARGET GENES

Gene Name	Promoter Species	Sequence	Promoter Position	EMSA NE, IVT	Deletion and mutation constructs	Co-Transfection of SF-1	Notes	Reference(s)
αGSU	Mouse	CTTGAGGTCA	-222	αT3, Y1, MA-10				(Ingraham et al., 1994)
3β-HSD2, type II 3β-hydroxysteroid dehydrogenase	Human	TTCAAGGTAA	-64	H295R	H295R,HeLa	CV-1, H295R, HeLa		(Leers-Sucheta et al., 1997)
ACTH Receptor, MC2 receptor	Human	CCCAAGGTCC	-35	Y1,bovine and human adrenal, IVT	Y1			(Marchal et al., 1998; Naville et al., 1997)
ACTH Receptor, MC2 receptor	Mouse	CTCAAGGTTA	-25	Y1	Y1	L cells		(Cammas et al., 1997)
Aldose reductase-like gene (AKR1B7)	Mouse	AGAAAGGTGT ATGTAGGTCA CACAAGGTCA GAACAGGAAC	-503A -503B -458 -102	Y1,IVT	Y1	CV-1	3 SF-1 binding sites in -510 fragment. This fragment can drive expression in adrenal cortex of transgenic mice.	(Aigueperse et al., 2001; Martinez et al., 2003; Val et al., 2004)
Aldosterone synthase (AS, Cyp11b-2)	Mouse	ACCAAGGTCT	-323	Y1,Bovine adrenal,SF-1 GST fusion				(Lala et al., 1992)
Aromatase (Cyp19)	Rat	CCCAAGGTCA	-82	R2C, rat ovary, SF-1 GST fusion	R2C		Concatamers of binding site/R2C	(Fitzpatrick and Richards, 1994; Lynch et al., 1993)
Cyp17	Bovine	GTCAAGGAGA	-69	Y1	Mutations to concatamer/Y1	Y1	Transfections used 4 copies of binding site. Binding site used TGAGCATTAACATAAAGTCAAGGAGAAGGTCAGGG	(Bakke and Lund, 1995)
Cyp17	Human	GTCAAGGTGA	-57	H295R, IVT	H295R	H295R		(Sewer et al., 2002)
Cyp21 (21-OHase)	Bovine	CCAAGGCCA CCAAGGCCA	-174 -166					(Lala et al., 1992)
Cyp21 (21-OHase)	Mouse	AGAGAGGTCA TCCAAGGCTGA GCAAAGGTCAG	-215 -154 -82	Y1, Bovine adrenal, SF-1 GST fusion				(Lala et al., 1992)
Dax-1	Human	ACCGAGGTCA	-110	IVT				(Burris et al., 1995)

Dax-1	Mouse	GTAGAGGTCA TTGCAGGTCA GTCATGGCCA GACAAGGGCG	-330 -122 -118 -80	Y1	Y1, R2C	CV-1	-122/-118 is a compound SF-1 site (CGAGGTCATGGCCA)	(Kawabe et al., 1999)
FSH receptor	Mouse	GCCAAGGACT	-1369	mLTC-1, KK-1,SF-1 transfected HEK293	HEK293, mLTC-1	HEK293, mLTC-1		(Levallet et al., 2001)
FSHβ	Mouse	GCGAAGGTAA TACAAGGTGA	-342 -239	αT3,Lβ T2	LβT2		SF-1 and NF-Y proteins physically interact. FSHβ promoter is not active in αT3 cells.	(Jacobs et al., 2003)
Gonadotropin II β	Salmon	ATCAAGGTCC GTAGAGGTCA	-354 -164	IVT	HeLa, GH4C1	HeLa, GH4C1	SF-1 and ER have synergistic activation on the promoter	(Drean et al., 1996)
Gonadotropin-Releasing Hormone Receptor	Human	CTCAGGGACA	-134	αT3,IVT	αT3	SKOV-3, Cos-7, αT3	Antisense SF-1 mRNA reduced the hGNRHR promoter activity in αT3 cells.	(Ngan et al., 1999)
Hydroxymethylglutaryl CoA (HMG-CoA)	Hampster	CTGAAGGTCA	-366	MA-10, IVT	CV-1, MA-10, mutation only	CV-1, MA-10		(Mascaro et al., 2000)
Inhibin α	Rat	CTCAGGGCCA	-138	tsa cells transfected with SF-1	tsa	tsa, GRMO2	cAMP pathway and SF-1 act synergistically on the promoter.	(Gummow et al., 2003; Ito et al., 2000)
INSL3, relaxin like factor	Mouse	TTCAAGGTCC GTCACGGTCA GCCAAGGCCC	-147 -115 -65	mLTC-1	mLTC-1, KK-1,MSC-1, HEK293	HEK293	-65 site highest affinity.Dax-1 was able to compete for binding in EMSA and reduce activation in transfections	(Koskimies et al., 2002)
Luteinizing Hormone β-Subunit	Rat Cow Pig Sheep Horse Human	GACAAGGTCA GACAAGGTCA CCAAGGTCA ACAAGGTCA GACAAGGTCA GACATGGCCA	-127 -121 -124 -129	IVT	CV-1, GH3	CV-1, GH3	SF-1 and Egr-1 can act synergistically on the LHβ promoter. Protein/protein interaction by GST pull down	(Halvorson et al., 1998)
Luteinizing Hormone β-Subunit	Rat Cow Pig Sheep Horse Human	GCAAGGCCT GCAAGGCCG GCAAGGCCA GCAAGCGCCG GCAAGGCCA GCAAGGCCA	-59	IVT, αT3	CV-1, GH3	CV-1, GH3	The SF-1 binding site is important for LHβ promoter activity in transgenic mice	(Halvorson et al., 1998; Halvorson et al., 1996; Keri and Nilson, 1996)
Luteinizing Hormone β-Subunit	Sheep	GACAAGGTCA	-127	Sheep pituitary				(Brown and McNeilly, 1997)
Müllerian Inhibiting Substance	Mouse	CCCAAGGTCA	-97	Rat P15 testis	Primary rat Sertoli cells	Primary rat Sertoli cells	Loss of SF-1 binding site prevented proper expression of MIS/GH transgene	(Giuili et al., 1997)

Műllerian Inhibiting Substance	Mouse Human Rat Bovine	CCCAAGGTCA CCCAAGGTCG CCCAAGGTCA TTCAAGGTCA	-95 -97 -91 -95	Bovine Adrenal, Rat fetal testis, IVT,R2C,Y1	Cos-7, NT2/D1	Cos-7	Direct protein-protein interaction between SF-1 and Sox9 co-ordinates Mis expression	(de Santa Barbara et al., 1998; Hatano et al., 1994; Shen et al., 1994)
Műllerian Inhibiting Substance Receptor II	Human	TCCAAGGTCA	-268	SF-1 GST fusion, NT2/D1, HeLa transfected with SF-1	NT2/D1	HeLa		(de Santa Barbara et al., 1998)
Műllerian Inhibiting Substance Receptor II	Rat	TCCAAGGTCA GCCAAGGTCC	-250 -200	R2C	R2C		CCAAGGTCA Site binds SF-1 with higher affinity than CCAAGGTCC. -200 site not present in human promoter	(Teixeira et al., 1999)
Neuronal Nitric Oxide Synthase	Mouse Rat Human Rabbit	GTCAAGGTTG GTCAAGGTTG GTGATGGTTG GCAATGGCTG	-208 -207 -248 -230	αT3	αT3, 3T3	αT3, 3T3	DAX-1 can inhibit promoter activity in a dose dependent manner.	(Wei et al., 2002)
NMDA receptor subunit NR2C	Mouse	CCCAAGGTCA	-252	IVT	3T3, HT-4	HT-4		(Pieri et al., 1999)
Oxytocin	Bovine	TCCAAGGTCA	-176	Bovine corpus luteum		TM4	COUT-TF also binds this site	(Wehrenberg et al., 1994)
Prolactin, Promoter 1	Rat	GCCAAGGTCA	-687	mLTC, rat ovarian, rat granulosa	mLTC		This binding site is not conserved in the mouse prolactin P1 promoter	(Hu et al., 1997; Hu et al., 1998)
Scavenger receptor, class B, type I (SR-BI/CLA-1)	Human	CCCAAGGCTG	-76	Y1, SF-1 GST fusion	Y1			(Cao et al., 1997)
Side Chain Cleavage (SCC,Cyp11a)	Bovine	GGAAGGTCA CCAAGGCTT TCAAGGCTA	-835 -820 -47					(Lala et al., 1992)
Side Chain Cleavage (SCC,Cyp11a)	Mouse	GGGGAGGTCA CTCAAGGCTA	-79 -47	Y1, Bovine adrenal, SF-1 GST fusion				(Lala et al., 1992)
Side Chain Cleavage (SCC,Cyp11a)	Rat	GGGGAGGTCA CTCAAGGCTA	-79 -51	Rat ovary, Rat Granulosa cells, SF-1 GST fusion				(Clemens et al., 1994)

SRY	Human	TAAAAGGTCA	-322	NT2/D1, SF-1 transfected HeLa cells, SF-1 GST fusion	NT2/D1	NT2/D1	Phosphorylated SF-1 has reduced affinity to binding site compared to unphosphorylated protein	(de Santa Barbara et al., 2001)
SRY	Pig	TCCAAGGTTA TTTAAGGTTA	-1369 -290	IVT	PGR 9E11 cells	CV-1		(Pilon et al., 2003)
steroid-11-beta-hydroxylase(Cyp11b1)								
Steroidogenic acute regulatory protein (StAR)	Bovine	ACCAAGGCTG GTCAAGGCAA ACCAAGGATA	-1190 -240 -100	Bovine corpus luteum, bovine adrenal cortex, IVT	HeLa, deletion only	HeLa	-240 and -100 sites were the most important in the transfection assays	(Rust et al., 1998)
Steroidogenic acute regulatory protein (StAR)	Human	TTCAAGGTCA GTCAAGGTCA	-926 -105	SF-1 GST fusion, SF-1 transfected Cos-1 cells	BeWo	BeWo, Cos-1, HeLa, SK-OV-3		(Sugawara et al., 1996)
Steroidogenic acute regulatory protein (StAR)	Mouse	GCCAAGGTGG TGGAAGGCTG	-135 -42	Y1	Y1			(Caron et al., 1997)
Steroidogenic acute regulatory protein (StAR)	Rat	TTCAAGGCCG TTCAAGGCCA GTCAAGGATG	-746 -455 -106	SF-1 GST fusion	Y1, HTB9	Y1, HTB9		(Sandhoff et al., 1998)

APPENDIX 2 CELL LINES USED TO STUDY SF-1 FUNCTION

Cell Line	Cell Type	Express SF-1
αT3	Mouse pituitary gonadotrophes (immature)	Yes
GRMO2	Granulosa cells	Yes
H295R	Human adrenocortical tumor	Yes
KK-1	Mouse granulosa cells	Yes
LβT2	Mouse pituitary gonadotrophes (mature)	Yes
MA-10	Mouse Leydig	Yes
mLTC-1	Mouse Leydig	Yes
MSC-1	Mouse Sertoli cells	Yes
NT2/D1	Human testicular embryonic carcinoma	Yes
R2C	Rat Leydig	Yes
TM3	Mouse Leydig	Yes
Y1	Mouse adrenal cortical	Yes
3T3	Mouse fibroblast	No
BeWo	Choriocarcinoma	No
Cos-1	Monkey kidney, SV40 transformed	No
Cos-7	Monkey kidney, SV40 transformed	No
CV-1	Monkey kidney	No
F9	Mouse teratocarcinoma	No
GH3	Somatolactotroph	No
GH4C1	Rat pituitary	No
HEK 293	Human embryonic Kidney	No
HeLa	Human cervical carcinoma	No
HTB9	Human bladder carcinoma	No
Jeg-3	Human choriocarcinoma	No
L cells	Mouse fibroblast	No
PC-12	Rat pheochromocytoma	No
SKOV-3	Human ovarian carcinoma	No
tsa	Human embryonic kidney	No

VITAE

Nancy Ruth Stallings was born in Los Alamos, New Mexico, on August 18, 1971, the second child of Richard Dale Stallings and Judith Ann Graves Stallings McGuire. She graduated from Los Alamos High School in June 1989. Nancy attended Trinity University in 1989-1990 and then transferred to the University of New Mexico for the remainder of her college education. She received the degree of Bachelor of Science with a double major in Sociology and Biology in May 1994. In December 1994, she married Joseph Strohl of Albuquerque, New Mexico. From June 1994-August 1997 Nancy was employed as a research technician at The University of Texas Southwestern Medical Center working for Drs. James Griffin, Jean Wilson and Michael McPhaul. In August 1997 she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas, choosing Dr. Keith Parker as her Ph.D advisor in June 1998. She was awarded the degree of Doctor of Philosophy in April, 2005.

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