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Two Regions Within the Proximal Steroidogenic Factor 1 Promoter Drive Somatic Cell-Specific Activity in Developing Gonads of the Female Mouse[1]

Liying Gao,[4] Youngha Kim,[7] Bongki Kim,[3,5,7] Stacey M. Lofgren,[8] Jennifer R. Schultz-Norton,[9] Ann M. Nardulli,[6] Leslie L. Heckert,[10] and Joan S. Jorgensen[2,7]

Departments of Veterinary Biosciences,[4] Animal Sciences,[5] and Molecular and Integrative Physiology,[6] University of Illinois at Urbana-Champaign, Urbana, Illinois
Department of Comparative Biosciences,[7] University of Wisconsin, Madison, Wisconsin
IVF Institute of Chicago,[8] Chicago, Illinois
Department of Biology,[9] Millikin University, Decatur, Illinois
Department of Molecular and Integrative Physiology,[10] University of Kansas Medical Center, Kansas City, Kansas

ABSTRACT

Targets of steroidogenic factor 1 (SF1; also known as NR5A1 and AD4BP) have been identified within cells at every level of the hypothalamic-pituitary-gonadal and -adrenal axes, revealing SF1 to be a master regulator of major endocrine systems. Mouse embryos express SF1 in the genital ridge until Embryonic Day 13.5 (E13.5). Thereafter, expression persists in the male and is substantially lower in the female gonad until birth. We hypothesize that the sexually dimorphic expression of *Sf1* during gonadogenesis is mediated by sex-specific regulation of its promoter. To investigate dimorphic regulation within the fetal gonad, we developed an experimental strategy using transient transfection of E13.5 gonad explant cultures and evaluated various *Sf1* promoter constructs for sexually dimorphic DNA elements. The proximal *Sf1* promoter correctly targeted reporter activity to SF1-expressing cells in both XY and XX gonads. Stepwise deletion of sequences from the *Sf1* promoter revealed two regions that affected regulation within female gonads. Mutation of both sequences together did not cause further disruption of reporter activity, suggesting the two sites might work in concert to promote activity in female somatic cells. Results from gel mobility shift assays and fetal gonad-chromatin immunoprecipitation showed that TCFAP2 binds to one of the two female-specific sites within the proximal promoter of *Sf1*. Together, we show that transient transfection experiments performed within developing testes and ovaries are a powerful tool to uncover elements within the *Sf1* promoter that contribute to sex-specific expression.

AP2, gonad, ovary, promoter, Sf1, *steroidogenic factor 1, transfection*

INTRODUCTION

Nuclear receptor subfamily 5, group A, member 1 (NR5A1), also known as steroidogenic factor 1 (SF1) or adrenal 4-binding protein (AD4BP), is a transcription factor that is a member of the nuclear hormone receptor family [1, 2]. Disruption of *Sf1* in mice results in adrenal and gonad agenesis in both sexes, thereby implicating it in early organogenesis [3–5]. These organs form normally at first but then disappear because of increased cell death by apoptosis, demonstrating a key role for SF1 in cell survival [3]. Haploinsufficiency of SF1 is permissive for testis development with a 24-h delay in expression of both fetal Leydig and Sertoli cell markers [6]. Further studies using targeted mutagenesis of *Sf1* in the ventromedial hypothalamus, gonadotrophs, Leydig cells, and granulosa cells have confirmed its intrinsic function in these tissues [7–9]. The loss of *Sf1* within Leydig and granulosa cells highlights the importance of *Sf1* in the development of the gonads and in steroidogenesis and fertility in adult mice [7]. In humans, a variety of *SF1* mutations have been identified in both XX and XY patients that result in a range of phenotypes, including gonad agenesis and sex reversal, infertility, and hypospadia [10–20].

In the mouse, all embryos express SF1 in the genital ridge until approximately Embryonic Day 13.5 (E13.5). As the male develops testicular cords, SF1 is expressed in Sertoli and interstitial cells, and expression continues in these cells throughout development. This persistent expression coincides with production of androgens and factors required for degradation of the Müllerian duct. In contrast, *Sf1* transcripts in female gonads decrease starting after E13.5 and disappear until birth, when primordial follicles begin to form [21, 22].

Studies on the proximal promoter region have identified elements and factors that contribute to *Sf1* activity in primary Sertoli cells and cells derived from the adrenal gland (Y1 adrenocortical), pituitary (αT3–1 and LβT2 gonadotroph), and testis (MA-10 Leydig, MSC-1, and TM4 Sertoli). These reports have determined that basal transcription of *Sf1* depends on the first 110 base pairs (bp) of the promoter, a region that is highly conserved among different species, and have established the importance of an E-box, a CCAAT box, and an Sp1 site in this region (Fig. 1) [23–27]. These elements bind ubiquitous factors including upstream stimulatory factor 1 (USF1) and USF2; NF-Y; and SP1, SP2, and SP3 [23–26, 28]. Conservation of the proximal promoter across mammalian species suggests common regulation. Recently, additional elements have been identified and include sequences that bind SOX9, WT1, and LHX9, each of which plays a significant part in gonad development (Fig. 1) [29, 30]. Of the known factors that bind the *Sf1* promoter, only SOX9 exhibits cell-specific expression in developing gonads, suggesting its potential role for regulating *Sf1* in Sertoli cells. WT1 and LHX9 are expressed

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[2]Correspondence: Joan S. Jorgensen, University of Wisconsin, Department of Comparative Biosciences, 2015 Linden Dr., Madison, WI 53706. FAX: 608 263 3927; e-mail: jsjorgensen@wisc.edu
[3]Current address: Bongki Kim, Department of Surgery, Children's Hospital, Harvard University, Boston, MA 02115.

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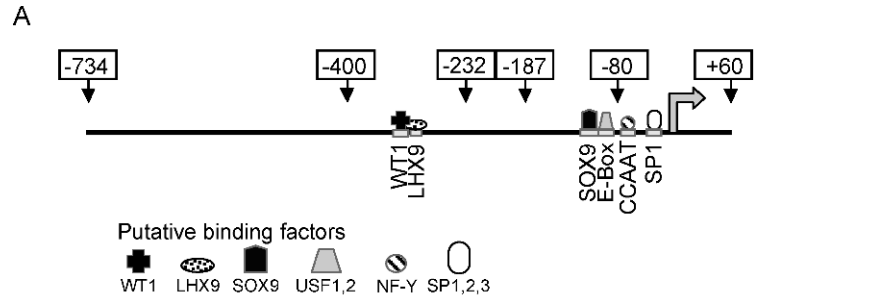
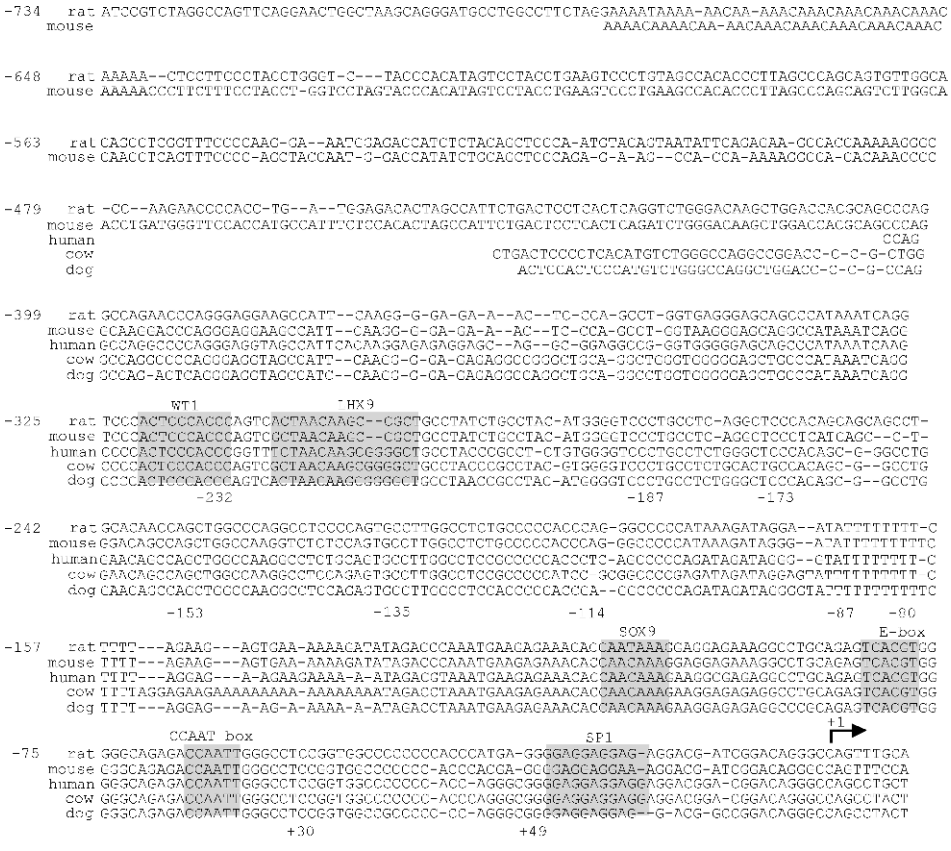


FIG. 1. The −734/+60 bp *Sf1* promoter. **A**) Reported binding sites and corresponding putative binding factors of the −734/+60 bp *Sf1* promoter are shown with approximate numbering based on +1 transcription start site of rat *Nr5a1*. **B**) Phylogenetic analysis was performed on the *Sf1* promoter to compare mouse, human, cow, and dog to rat sequences. Sequences were obtained from the Ensembl database and aligned using MULAN sequence analysis (http://mulan.dcode.org/). Numbering is based on the rat sequences, and known binding sites are labeled and shaded in light gray.

B



very early in development of the bipotential gonad and contribute to *Sf1* expression at this time [30]; however, they are unlikely to contribute to cell or sex specificity of *Sf1* expression during sex differentiation.

Currently, there are no appropriate cell lines to investigate sex- and cell-specific regulation of genes expressed in the developing gonad. Transgenic mouse models used to examine tissue- and cell-specific expression of *Sf1* has led to the discovery of enhancer sequences within intron sequences and at considerable distances from the *Sf1* transcription start site (TSS) that contribute to its expression in the ventromedial hypothalamus, pituitary gonadotroph, or adrenal cortex [31–33]. To investigate the regulation of *Sf1* in a way that is both physiologically relevant and subject to quantitative analysis, we developed a strategy to quantify promoter activity within gonad explant cultures based on a previously described electroporation technique [34]. An *Sf1* promoter (−734/+60 bp), which included sequences sufficient to target reporter activity to the bipotential gonad in transgenic mice [30], was used to compare reporter activity between male and female gonads at E13.5, at which time, *Sf1* expression and its target genes begin to exhibit cell- and sex-specific characteristics. Electroporation of the −734/+60 bp *Sf1* promoter linked to luciferase or EGFP (enhanced green fluorescent protein) faithfully targeted reporter activity to SF1-expressing cells and facilitated quantification of promoter activity from gonad explant cultures. We transfected several mutated versions of the proximal *Sf1* promoter and discovered that disruption of two specific regions significantly decreased reporter activity in explant cultures of female gonads. Sequence analysis and binding assays suggest that TCFAP2A/C (otherwise known as AP2α/γ) interacts with one of these regions. Altogether, the results of these studies suggest that elements within the proximal promoter contribute to regulation of *Sf1* that is

TABLE 1. Subcloning primers.

Primer		Primer sequence[a]	
−114bp XhoI	−114bp	5′ GGGGCTCGAGCCAATAAAGGAGGAGAAAGG 3′	−95bp
+60bp HindIII	+60bp	5′ GGGGAAGCTTCTATCGGGCTGTCAGGAACT 3′	+41bp
μ112bp	−124bp	5′ AGAGAAACACCA**GAATTCGAGGAGAAGGC** 3′	−96bp
μ105bp	−117bp	5′ ACACCAATAAAG**GAATTCAAAGGCCTGCAG** 3′	−88bp
μ99bp	−111bp	5′ ATAAAGGAGGAG**GAATTCCTGCAGAGTCAC** 3′	−82bp
μ93bp	−105bp	5′ GAGGAGAAAGGC**GAATTCAGTCACGTGGGG** 3′	−75bp

[a] Bold indicates mutated sequence.

specific to the developing gonad during the time of sex differentiation.

MATERIALS AND METHODS

Animals

Mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in AAALAC-accredited facilities, and the University of Wisconsin-Madison Animal Care and Use Committee approved all the procedures. CD1 outbred mice were purchased from Charles River Laboratories for timed breedings. The presence of a vaginal plug was designated as Embryonic Day 0.5 (E0.5). Gonads were isolated from embryos harvested at E13.5 or E15.5.

Promoter Constructs and DNA Preparation

Sf1 promoter constructs derived from rat sequences, including −734/+60 bp, −232/+60 bp, −187/+60 bp, −173/+60 bp, −153/+60 bp, −135/+60 bp, −87/+60 bp, −80/+60 bp, −734/+49 bp, −734/+30 bp, −734/+10 bp, and μE-box, were generated by the laboratory of Leslie L. Heckert (University of Kansas Medical Health Science Center) and described elsewhere [23, 28]. The −114/+60 bp construct was made by amplifying the fragment by PCR from the −734/+60 bp pGL3 construct using primers with XhoI and HindIII linkers (see Table 1) and placing the fragment into pGL3-Basic digested with XhoI/HindIII. Block mutations were made within the −734/+60 bp promoter using PCR primers that contained a block replacement of six base pairs in four mutations including μ112 (replaced −112 to −107 bp), μ105 (replaced −105 to −100 bp), μ99 (replaced −99 to −94 bp), and μ93 (replaced −93 to −88 bp). Primers for block mutations are shown only in the sense direction; each primer has its positions noted relative to the major transcriptional start site at +1 based on the rat *Sf1* promoter sequence (see Table 1) [28]. Primers containing the block mutation for each sequence were used to make mutant constructs with the QuikChange II site-directed mutagenesis kit as directed in the manufacturer's protocol (Stratagene). This kit was also used to make −734/+30 bp μ93 (−734/+30 bp *Sf1* luc mutated with μ93 primers). Also, −734/+60 bp, μE-box, μSOX9, μ112, −114/+60 bp, and −80/+60 bp *Sf1* promoters were shuttled to pEGFP-1 (Clontech) using XhoI/HindIII restriction sites. All the primers were ordered from Integrated DNA Technologies. Two separate clones of each mutation were sequenced and used for transfection experiments. All the plasmid DNAs were prepared from overnight bacterial cultures using Qiagen DNA plasmid columns according the manufacturer's protocol (Qiagen).

Gonad Explant Culture and Transient Transfection Analysis

Transient transfection assays in urogenital ridge explant cultures were based on previously reported methods of the explant culture system [34]. The sex of the gonad tissue was determined by characteristic findings of a coelomic vessel and testicular cords in the male and the lack of these in the female. Urogenital ridges were harvested from embryos at E13.5 or E15.5 and immediately placed in 7 μl of Dulbecco phosphate-buffered saline (PBS; Sigma D8537) containing a cocktail of 4 μg/μl SF1-luciferase plus 2 μg/μl SV40-Renilla luciferase. Next, approximately 0.2 μl of the DNA mixture was injected into the gonad in three different sites. An additional aliquot of 25 μl of sterile PBS was placed on the gonad for electroporation. Immediately thereafter, five square electrical pulses of 65 V, 50 msec each at 100-msec intervals, were delivered through platinum electrodes from an electroporator. After electroporation, urogenital ridges were placed back into the culture for 24 h. Explant cultures were maintained at 37°C with 5% CO_2/95% air on a 1.5% agar block or in 50 μl of Dulbecco minimal Eagle medium (DMEM) supplemented with 10% FCS (fetal calf serum) and 50 μg/ml ampicillin [35]. Transfected gonad explants were harvested and processed for either dual luciferase assays or immunohistochemistry.

The electroporator was hand assembled by the electronics shop at the University of Illinois; schematic plans can be made available by request. Needles for injection are made from thin-walled glass capillaries (4 inches long, 1.0 mm wide, TW100F-4; World Precision Instruments) using a flaming brown micropipette puller (P80/PC; Sutter Instrument) with settings of heat = 520, velocity = 80, pull = 5, and time = 60.

The dual luciferase assay was performed as follows: gonads were harvested, washed three times with PBS, and then placed in 100 μl of passive lysis buffer (Promega) and manually disrupted with a 26-g needle. The explant cultures were subjected to three freeze/thaw cycles to optimize cell lysis. Following cell lysis, 20 μl of cell lysate was assessed for reporter activity using the dual reporter detection system according to manufacturer's recommendations (Promega). For each experiment, each construct was injected in at least three gonads of each sex, and each experiment was repeated at least three times. At least two independent clones of each promoter construct were injected. The transfection data were represented as the firefly luciferase/Renilla luciferase activity of each construct, which was normalized to the firefly luciferase/Renilla luciferase activity of pGL3-Basic promoter construct.

Gonads were transfected with pEGFP-N1 (Clontech) or SF1-pEGFP constructs (4 μg/μl) for reporter localization studies. After transfection, they were incubated overnight, harvested, fixed in 4% paraformaldehyde at 4°C overnight, and then frozen in 3:1 OCT:20% sucrose for sectioning and immunohistochemistry.

The αT3–1 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Sigma). Twenty-four hours before transfection, 180 000 cells were plated per well in 6-well plates. Cells were transfected with the indicated DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. Luciferase reporter constructs (1.25 μg/well) were cotransfected with SV40-Renilla (0.25 μg/well). Dual luciferase activity was measured using the dual reporter detection system according to the manufacturer's protocol (Promega). The values were averaged over a minimum of three independent experiments.

RNA Isolation and Quantitative PCR

Quantitative real-time PCR (QPCR) was performed as previously reported [36]. QPCR was performed at least three times with at least three different biological replicates of pooled RNA from gonads. The primers are listed in Table 2.

Immunohistochemistry

Transfected urogenital ridges (gonad and mesonephros) were harvested after 24-h culture and fixed in 4% paraformaldehyde at 4°C overnight. Tissues were washed in PBS, then cryoprotected with a series of sucrose gradient washes, and finally frozen in a 3:1 OCT:20% sucrose cocktail. Frozen sections (10 μm) were incubated with anti-GFP antibody (A11122; Invitrogen) and compared to SF1-expressing cells by incubation with rabbit anti-SF1 (PA1–800; Thermo Fisher Scientific) or to germ cells using anti-TRA98 antibody (BAM-73–003; Cosmo Bio USA); rabbit anti-laminin (L9393; Sigma) was used to demarcate the testis cords. Samples were incubated overnight at 4°C and then treated with fluorescently conjugated secondary antibodies (Jackson Laboratories) for detection. Fluorescent images were captured using a Spot camera (Diagnostic Images, Inc.) installed on a Nikon Eclipse E600 microscope (Nikon Instruments Inc.). NIH-supported Image J software (http://rsbweb.nih.gov/ij/) was used to merge fluorescent wavelengths.

Isolation of PECAM+ AND PECAM– Cell Populations from Gonads Using Magnetic Beads

Germ and endothelial cells (PECAM+ [platelet endothelial cell adhesion molecule positive], also known as CD31+) were separated from the

TABLE 2. Quantitative PCR primers.

Gene	Forward primer	Reverse primer
Tfap2a	5′ TCAACCGACAACATTCCGATCCCA 3′	5′ TGAAGTGGGTCAAGCAACTCTGGA 3′
Tfap2c	5′ ACGTCTCTCGTGGAAGGTGAA 3′	5′ GGAACTCAGCTTCGCAGACAT 3′
Ets2	5′ CGGCGCGATGAATGACTTTGGAAT 3′	5′ AGAAGGGAGCACAGCAAACAGAGA 3′
Cebpb	5′ TGATGCAATCCGGATCAAACGTGG 3′	5′ TTTAAGTGATTACTCAGGGCCCGGCT 3′
Cebpg	5′ GAGGCGCAGGTACATGTGAAGATT 3′	5′ CTTGCTCATTTGGGCACGTTCCTT 3′
Sf1	5′ TGCACTGCAGCTGGACCGCCAGGAGTT 3′	5′ AGGGCTCCTGGATCACCTAATGCAAGGA 3′
Ddx4	5′ GAGATTGCCTTCAGTACCTATGTG 3′	5′ GTGCTTGCCCTGGTAATTCT 3′

remaining somatic cells (PECAM−, also known as CD31−) in the genital ridge by magnetic cell sorting using positive selection. Briefly, E13.5 genital ridges were incubated with collagenase type I (C2674; Sigma) for 1 h at 37°C in DMEM containing 10% FCS. Cells were dispersed, washed, and resuspended in 100 μl of binding buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA) containing phycoerythrin-conjugated (PE) rat anti-mouse CD31 antibody (BD Biosciences) and incubated for 10 min at 4°C. The cells were washed and then incubated with anti-PE microbeads (130-048-801; Miltenyi Biotec) for 15 min at 4°C. Magnetic separation was carried out according to the manufacturer's instructions using the MS column and OctoMACS separator (Miltenyi Biotec). The PECAM− cells were collected from the flow through to obtain somatic cells. RNA was extracted with the RNeasy Micro Kit (Qiagen) and QPCR assays were carried out using the SYBR Green I Kit (Applied Biosystems). The comparative Ct method was used to calculate the fold difference of PECAM− values compared to the PECAM+ control for each gene [37]. At least three biological replicates were assayed, and each gene was analyzed in triplicate.

Electrophoretic Mobility Shift Analysis

TCFAP2 proteins were synthesized using TnT in vitro transcription/translation kits with a biotinylated lysine label (TnT-coupled reticulocyte lysate system with transcend nonradioactive translation detection system; Promega). Expression vectors for human TCFAP2A and TCFAP2C were generously provided by Ronald A. Weigel (University of Iowa) [38]. The negative control was made using the pcDNA3.1 vector. Lysine-labeled protein products were verified using colorimetric analysis as described in the manufacturer's protocol (data not shown). The oligodeoxynucleotides used for the gel shifts are described below. Complementary single-strand oligodeoxynucleotides were annealed and then end-labeled using [γ^{32}P]ATP and T4 polynucleotide kinase.

Sequences of oligodeoxynucleotides used for gel shift assays are shown in sense strand with mutations in bold type. The positions given are relative to the major transcriptional start site at +1 based on the rat *Sf1* promoter sequence [28]. Wild type −105/−76 bp *Sf1* promoter: 5′ GAGGAGAAAGGCCTGCA GAGTCACGTGGGG 3′. Mutant −105/−76 bp *Sf1* promoter: 5′ GAGGA GAAAGGC***GAATTC***AGTCACGTGGGG 3′.

Binding reactions were performed at a final volume of 20 μl in EMSA buffer, which includes 10 mM Tris, pH 7.5, 5 mM NaCl, 0.5 mM dithiothreitol, 0.005% NP-40, 1 μg poly(dIdC), and 10% glycerol. Reaction mixtures were preincubated for 15 min on ice in the presence of 1 μl protein lysate reaction, followed by the inclusion of 0.1 pmol of radiolabeled probe (~300 000 cpm/pmol). Unlabeled wild type or mutant oligodeoxynucleotides were incubated at 1× (i.e., 0.05 pmol), 10× (i.e., 1 pmol), and 100× (i.e., 10 pmol) concentrations for 15 min at room temperature followed by the addition of labeled probe. Alternatively, antibodies against nonspecific protein (anti PARP, sc-25780; Santa Cruz Biotechnology, Inc.) or TCFAP2A (sc-184X; Santa Cruz Biotechnology, Inc.) were incubated with the buffer-protein mix on ice for 10 min before labeled probe was added. Once the labeled probe was added, the mixture was incubated for 15 min at room temperature. DNA-protein complexes were resolved in low ionic strength polyacrylamide Tris-glycine gels for 1.75 h at 300 V in 4°C recirculating buffer. Gels were dried and analyzed by autoradiography.

Chromatin Immunoprecipitation Analysis

Gonads from embryos at E13.5 were harvested and treated with 1% formaldehyde for 20 min at 37°C followed by two washes with ice cold PBS, snap frozen, and stored at −80°C until enough material was accumulated for a chromatin immunoprecipitation (ChIP) analysis. Approximately 40–60 pairs of gonads were pooled and subject to a ChIP assay kit according to the manufacturer's protocol (17–295; Millipore). After sonication, protein concentrations were measured using the Bio Rad DC assay (500-0116; Bio Rad), and the ChIP assay was performed using 30 μg protein for the 10% input samples and 300 μg protein for the IP samples. One microgram TCFAP2A antibody was used to evaluate endogenous *Sf1* sequences (sc-184X; Santa Cruz Biotechnology, Inc.). Following elution of the antibody-protein-DNA complex, the cross-links were reversed, and the DNA was isolated and cleaned up using the Cycle Pure Kit (D6493–01; Omega Bio-Tek) and resuspended to a final volume of 50 μl. PCR was performed on 3–5 μl DNA using both standard and QPCR methods. QPCR was carried out in triplicate with primers specific to the region of interest. The percent recovery from the input was determined using the comparative Ct method, and this was then used to calculate the fold change from IgG or no antibody control [37]. *Sf1* primers (Table 3) were used to analyze −99/+87 bp sequences (promoter, covers −366/−69 bp relative to the TSS) and a random region downstream of the *Sf1* gene (downstream, covers 23 050/23 246 bp distal to TSS, or approximately 1200 bp distal to the 3′ end of the last exon). E-cadherin (*Cdh1*) sequences were used as a positive control (covers −144/+8 bp from TSS) [39]. ChIP experiments were repeated with at least four different biological replicates.

Statistical Analysis

Statistical analysis was performed using Student *t*-test, one-way, or two-way ANOVA as indicated with SigmaPlot 11.2 software (Systat Software, Inc.).

RESULTS

Electroporation Drives Plasmid DNA into All Cells of the Developing Gonad

To characterize the efficiency of the transfection technique in our laboratory, we injected and electroporated pEGFP-N1, which uses the immediate early promoter of cytomegalovirus (CMV) to drive ubiquitous expression of EGFP in explant gonads. Gonads were injected in three to four locations and then monitored for EGFP expression over time in culture. EGFP was detected as early as 4 h and maintained for at least 48 h posttransfection (Fig. 2A). Transfection efficiency appeared greatest in the immediate vicinity of DNA injection. Gonads transfected with pEGFP-N1 were also processed for immunohistochemistry using antibodies against GFP and a germ cell marker, TRA98, to assess cell-type localization of reporter activity. The results showed substantial expression of

TABLE 3. ChIP primers.

Gene	Forward primer	Reverse primer
Sf1 promoter	5′ GAAACTCCCAGCCTGGTGAGG 3′	5′ CTCTGCCCCCACGTGACT 3′
Sf1 downstream	5′ CAAGGCAATGCCCAGGCTTAAAGA 3′	5′ TTGCCACACTGTTCTATCTGGGCT 3′
Cdh1	5′ TGCCACCAACTACAGACAGG 3′	5′ ACACCAGTGAGCAGCGCAGA 3′

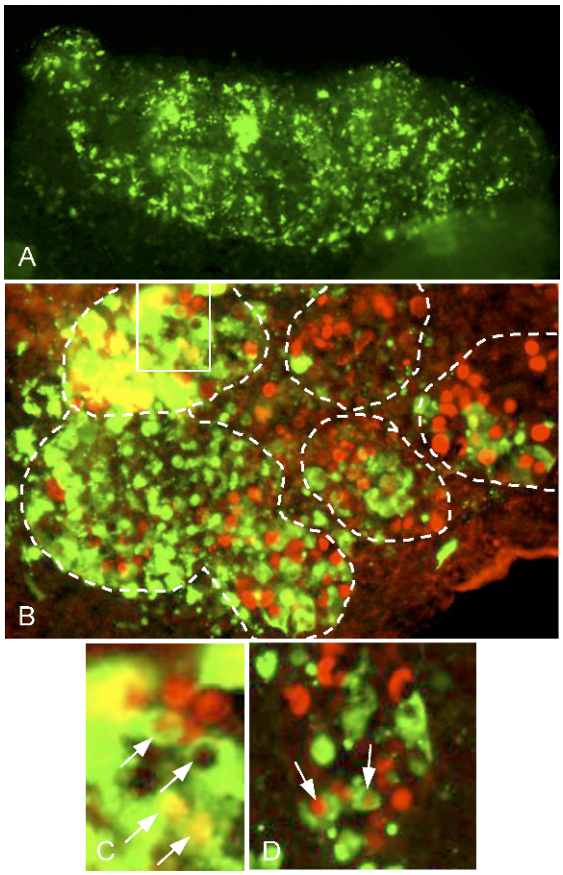


FIG. 2. Injection and electroporation of pEGFP-N1 into E13.5 gonads. **A**) The pEGFP-N1 plasmid was transfected into an E13.5 male gonad by microinjection and electroporation. After 24-h culture, the whole tissue was imaged using a fluorescent microscope to illustrate relative transfection efficiency. A representative example from several transfections is shown. **B**) E13.5 male gonads were transfected with pEGFP-N1 and then processed for immunohistochemistry with antibodies against GFP (green) and the germ cell marker, TRA98 (red) (dotted lines outline testis cords). **C**) Inset from the sample in (**B**) (box) labeled for GFP (green, cytoplasmic) and TRA98 (red, nuclear), arrows highlight colocalized cells that emit yellow fluorescence upon merging. **D**) E13.5 female gonad transfected with pEGFP-N1 and then processed for immunohistochemistry with anti-GFP (green) and anti-TRA98 (red); arrows point out colabeled cells. Original magnification ×5 (**A**) and ×20 (**B**, **C**, **D**).

EGFP in TRA98-labeled (germ) and -unlabeled (somatic) cells (Fig. 2, B–D).

The −734/+60 bp Sf1 *Promoter Is Active in Mouse Gonad Explant Cultures*

While considerable effort has been made to define cell-specific regulation of *Sf1* in adult cells, studies in embryonic tissues are limited. *Sf1* sequences that include 645 bp of the proximal promoter (−590/+85 bp) are sufficient to drive reporter activity to the appropriate cell types in embryonic gonads of transgenic mice [30]. Therefore, we anticipated that sequences that include −734/+60 bp would also promote activity within appropriate cell types of developing gonads. In addition, at E13.5, *Sf1* begins its progression from uniform expression in all somatic cells of the developing gonads to increases in Leydig cells and maintenance in Sertoli cells of male gonads, and to diminished levels in somatic cells of the female gonad [21, 40]. Thus, we reasoned that *Sf1* regulatory mechanisms would become cell- and sex-specific at this time. Male and female gonads were accordingly harvested from embryos at E13.5. The −734/+60 bp *Sf1* promoter linked to luciferase [23] and SV40-Renilla plasmids were injected into explant gonads followed by electroporation and cultured for 24 h. The results showed that luciferase activity was significantly elevated in both female and male gonads when compared to a promoterless control (pGL3-Basic). There was a trend toward higher reporter activity in male versus female gonads, but this difference was not statistically significant (Fig. 3A). Notably, there was no difference in reporter activity in female gonads when the results were compared between E13.5 and E15.5 when endogenous *Sf1* is low. The −734/+60 bp *Sf1* promoter was shuttled to an EGFP reporter (pEGFP-1) and used to localize promoter activity relative to SF1-expressing cells in transfected gonads. The *Sf1* promoter targeted EGFP activity to somatic cells marked by an anti-SF1 antibody in both female (Fig. 3, B and C, arrows) and male gonads (Fig. 3, D–G, with Leydig and Sertoli cells marked by arrowheads and arrows, respectively). Additional studies using a germ cell marker, TRA98, confirmed there was no spurious *Sf1*-EGFP activity in germ cells (data not shown). Together, these data indicate that transfection of the −734/+60 bp *Sf1* promoter into gonad explant cultures results in successful targeting of reporter activity to the appropriate cell types in developing gonads of both sexes and provides quantifiable activity. We also conclude that the −734/+60 bp *Sf1* promoter contains insufficient information to recapitulate loss of expression in female gonads.

The −734/+60 bp Sf1 *Promoter Requires E-Box but Not SOX9 Binding Sequences for Activity in Gonads from Both Sexes*

E-box sequences have been shown to be essential to *Sf1* promoter activity in all the models tested thus far [23–27], and the SOX9-binding element binds SOX9, the only known potential regulator that also exhibits sex-specific expression [29]. Therefore, we tested the significance of E-box and SOX9-binding sequences within the *Sf1* promoter in embryonic gonads by transfecting mutated constructs into gonad explant cultures. Similar to previous reports, mutation of the E-box-binding sequence caused a significant reduction in *Sf1*-driven reporter activity in both male and female gonads (Fig. 4A). While μE-boxμ-SF1-pEGFP activity was significantly lower in male and female gonads, no activity was detected in germ cells (data not shown). In contrast to reduced activity caused by a disrupted E-box sequence, mutation of the SOX9-binding site had no effect on reporter activity in gonads from either sex (Fig. 4B). Furthermore, the SOX9 mutant construct linked to EGFP localized reporter activity to both Leydig and Sertoli cells in transfected testes (Fig. 4C); no EGFP was detected in germ cells (Fig. 4, D and E). These data verify the importance of the E-box-binding sequence and suggest that SOX9 binding at the −110 bp site is not essential for *Sf1* promoter activity in male or female gonads during sex differentiation.

Two Regions of the −734/+60 bp Sf1 *Promoter Confer Activity in Female Gonads*

To determine whether there were any regions of the −734/+60 bp *Sf1* promoter that displayed sexually dimorphic activity, we transfected several deletion mutants into embryonic gonads. Stepwise deletions of the 5′ UTR (untranslated region) sequences in the *Sf1* promoter were assessed for sex-specific differences in activity. A significant decrease in

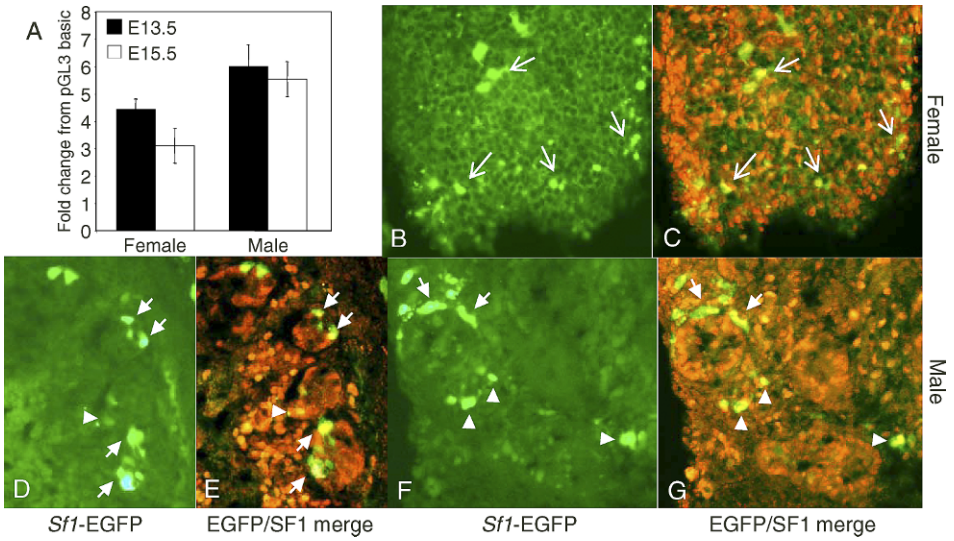


FIG. 3. The −734/+60 bp *Sf1* promoter can be transfected and is active in developing gonads. **A**) −734/+60 bp *Sf1*-driven luciferase reporter activity was detected in both male and female gonads at E13.5 and E15.5. Fold change from pGL3-Basic represents luciferase/Renilla activity of the *Sf1* promoter normalized to pGL3-Basic (promoterless control), which was set to 1.0 in gonads from each sex. Each construct was injected into at least three gonads of each sex, and each experiment was repeated at least three times. Data represent the mean ± standard error of the mean (SEM). Two-way ANOVA detected no differences between sex or time. **B**–**G**) The −734/+60 bp *Sf1* promoter drives EGFP to somatic cells in female gonads at E13.5 (arrows, **B** and **C**) and Leydig (arrowhead) and Sertoli (arrow) cells in male gonads (**D**–**G**). Examples of one transfected ovary and two testes and are shown with EGFP (green fluorescence, **B**, **D**, **F**) merged with anti-SF1 immunohistochemistry (rhodamine, **C**, **E**, **G**). Yellow coloring indicates colocalized expression. Original magnification ×20 (**B**–**G**).

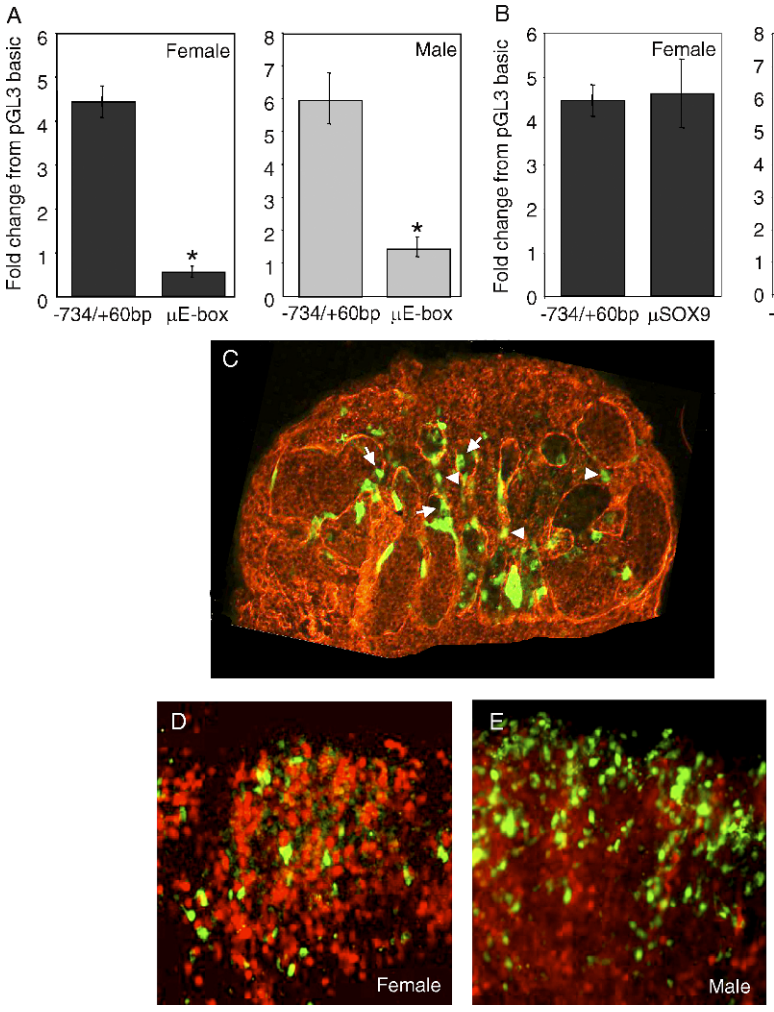
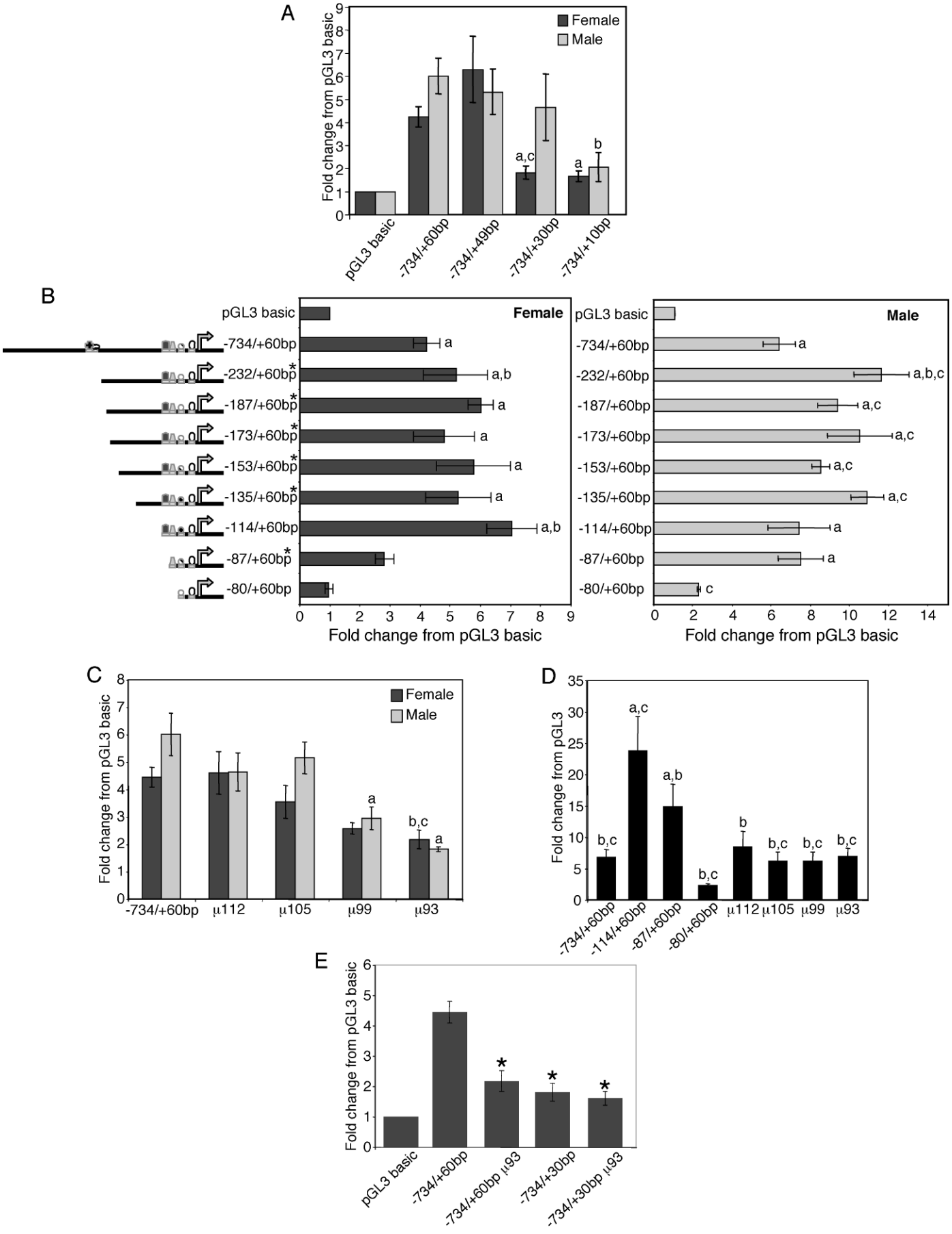


FIG. 4. The −734/+60 bp *Sf1* promoter requires E-box but not SOX9-binding sequences for activity in male and female gonads. **A**) A six base pair block mutation of the E-box within the context of the −734/+60 bp *Sf1* promoter disrupted luciferase activity in both male and female gonads. **B**) A block mutation of the SOX9 site within the −734/+60 bp *Sf1* promoter had no significant effect on reporter activity in gonads of either sex. In (**A**, **B**), each construct was injected into at least three gonads of each sex, and each experiment was repeated at least three times. Data represents mean ± SEM. Asterisks represent a significant difference from the −734/+60 bp promoter activity in male and female gonads (Student *t*-test, $P < 0.05$). **C**) Male gonads were transfected with μSOX9 −734/+60 bp SF1-pEGFP and processed for immunohistochemistry with laminin (red) to localize EGFP (green) reporter activity within the testis cords (arrows indicate transfected Sertoli cells; arrowheads, transfected Leydig cells). Female (**D**) and male (**E**) gonads were transfected with μSOX9 SF1-pEGFP and analyzed by immunohistochemistry using antibodies for EGFP (green) and germ cell marker TRA98 (red) at E13.5. Original magnification ×10 (**C**) and ×20 (**D**, **E**).



activity was detected for the −734/+10 bp promoter in both male and female gonads (Fig. 5A, asterisks). This result is consistent with previous studies performed in male-derived tissues, including primary Sertoli cells, the MSC-1 Sertoli cell line, and the MA-10 Leydig cell line [23]. A dramatic decrease in reporter activity specific to female gonads was detected when results were compared between the −734/+49 bp and −734/+30 bp *Sf1* promoters (Fig. 5A), suggesting this region is important for expression of *Sf1* in the female gonad.

Next, stepwise deletion of the proximal promoter uncovered a number of mutants with significant changes in reporter activity in both male and female gonads. Reporter activity of several promoters, −232/+60, −187/+60, −173/+60, −153/+60, −135/+60, and −87/+60 bp, was significantly higher in male versus female gonads (Fig. 5B). A significant decrease in activity was detected in both male and female gonads with the −80/+60 bp *Sf1* construct (Fig. 5B). This version of the *Sf1* promoter lacks the E-box binding sequence and corroborates our data from above (Fig. 4A). Sexually dimorphic reporter activity was detected between sequences −114 and −87 bp of the promoter, which was significantly decreased only in female gonads (Fig. 5B). Transfection of the −87/+60 bp *Sf1* promoter linked to EGFP was processed for immunohistochemistry to localize reporter activity. Results verified that all the reporter activity remained in somatic cells of both sexes (data not shown).

To examine the activity of the sequences between −114 and −87 bp more closely, we designed four mutant constructs in the context of the −734/+60 bp *Sf1* promoter. Each included a six base pair block replacement of sequences −112 to −107 bp (μ112), −105 to −100 bp (μ105), −99 to −94 bp (μ99), and −93 to −88 bp (μ93). *Sf1*-driven luciferase activity was significantly decreased with mutants μ99 and μ93 compared to the −734/+60 bp promoter in male gonads, and there was a trend toward decreased reporter activity with μ93 in female gonads (Fig. 5C). Activity of both μ99 and μ93 promoters were statistically decreased in gonads from both sexes at E15.5 (data not shown). The decrease in activity was similar in both mutants, suggesting that important factor(s) bind sequences that span the μ93 and μ99 mutations in female and male gonads.

We were surprised to detect a decrease in reporter activity by mutants μ99 and μ93 in male gonads because the 5′ deletion results comparing −114/+60 to −87/+60 bp constructs were not different. To assess whether the changes in reporter activity for these specific sequences was a global consequence of these specific mutants, we tested their activity in another model system that expresses SF1, the αT3–1 gonadotroph cell line. The results showed that both −114/+60 and −87/+60 bp *Sf1* reporter constructs are significantly increased over the −734/+60 bp *Sf1* promoter (Fig. 5D). Although the −80/+60 bp promoter was not statistically different from the −734/+60 bp construct, there appeared a definite decreasing trend. None of the block mutation constructs were different from the −734/+60 bp promoter, suggesting that the changes detected in male and female gonads were unique to the gonad explant system (Fig. 5D).

Taken together, these data imply that two distinct sequences within the −734/+60 bp promoter, including +30/+49 bp and −99/−87 bp, contain important information for activating the promoter in the female gonad while the −99/+87 bp region may also contribute to regulation in the male gonad. To determine the relative contribution of each region on female-specific activity, we performed transient transfection assays in female gonads with the μ93 block mutation made in the context of the −734/+30 bp *Sf1* promoter. We predicted that if the two mutations were independent, the combination of the two mutations would completely abrogate *Sf1* reporter activity. Both mutations in combination did not significantly decrease promoter activity further than −734/+60 bp μ93 or −734/+30 bp *Sf1* promoters alone suggesting that the two sequences might work together to stimulate *Sf1* promoter activity in the female gonad (Fig. 5E).

TCFAP2 Factors Bind −99/−87 bp Sequences in the Proximal Promoter of Sf1

Sequences between −99/−87 bp of the *Sf1* promoter are highly conserved among mammalian species. Only one base is different when rat, mouse, human, cow, and dog sequences were compared (Fig. 6A). In silico analysis was performed using both TESS (transcription element search system, http://www.cbil.upenn.edu/cgi-bin/tess/tess) and MULAN (multiple alignment of sequences across species, http://mulan.dcode.org/) comparative genomics search engines. Analysis of the −734/+60 bp (based on rat sequences) promoter uncovered a potential TCFAP2A/C-binding site that spanned from −95 to −87 bp (Fig. 6A) [41]. This binding site spanned both μ99 and μ93 mutations that were affected in the transient transfection analyses described above.

Relative expression of *Tcfap2a* and *Tcfap2c* was measured in male and female gonads over time and compared to the value obtained from female samples at E13.5 (Fig. 6B). Expression of *Tcfap2a* in female gonads was approximately 4-fold higher than in male gonads at both time points with expression at E15.5 substantially higher. Likewise, *Tcfap2c* expression in female gonads was over 3-fold higher than the male gonads at E13.5. By E15.5, however, female transcripts were dramatically decreased whereas male transcripts persisted (Fig. 6B).

FIG. 5. Two regions of the −734/+60 bp *Sf1* promoter harbor sequences required for activity in female gonads. **A**) Sequential deletions of the 5′ UTR were transfected into female (dark bars) and male (light bars) gonads at E13.5 and assessed for dual luciferase activity (a: $P < 0.01$ from −734/+49 bp female; b: $P < 0.01$ from −734/+60 bp male; c: $P = 0.08$ between sexes). **B**) Sequential deletions from the 5′ end of the −734/+60 bp *Sf1* promoter were transfected into female (dark bars, left panel) and male (light bars, right panel) gonads (two-way ANOVA, $^*P < 0.05$ difference between sex; a: $P < 0.02$ difference within sex from −80/+60 bp promoter; b: $P < 0.01$ difference within sex from −87/+60 bp promoter; c: $P < 0.03$ difference within sex from −734/+60 bp promoter). **C**) Six base pair block mutations were made within the context of the −734/+60 bp promoter starting at −112 bp to define the region of interest between −114 and −87 bp (six base pairs starting at each noted site were replaced with an EcoRI restriction digest site; see the sequences listed in *Materials and Methods*) and transfected into female (dark bars) and male (light bars) gonads at E13.5 (two-way ANOVA, a: $P < 0.008$ difference within male gonads from −734/+60 bp promoter; b: $P < 0.05$ difference within female gonads from μ112 construct; c: $P = 0.06$ difference within female gonads from −734/+60 bp promoter). **D**) The indicated promoters were transfected into αT3–1 gonadotroph cells to compare to gonad reporter activity (one-way ANOVA, a: $P < 0.05$ difference from −734/+60 bp promoter; b: $P < 0.03$ difference from −114/+60 bp promoter; c: $P < 0.05$ difference from −87/+60 bp promoter). **E**) The −93 bp block mutation was made in the context of the −734/+30 bp *Sf1* promoter construct and transfected into female gonads alongside the −734/+60 bp promoter, μ93 block mutation in context of −734/+60 bp promoter, and −734/+30 bp promoters (one-way ANOVA, $^*P < 0.001$ difference from −734/+60 bp promoter). Each construct was injected into at least three gonads of each sex, and each experiment was repeated at least three times. Data represents mean ± SEM.

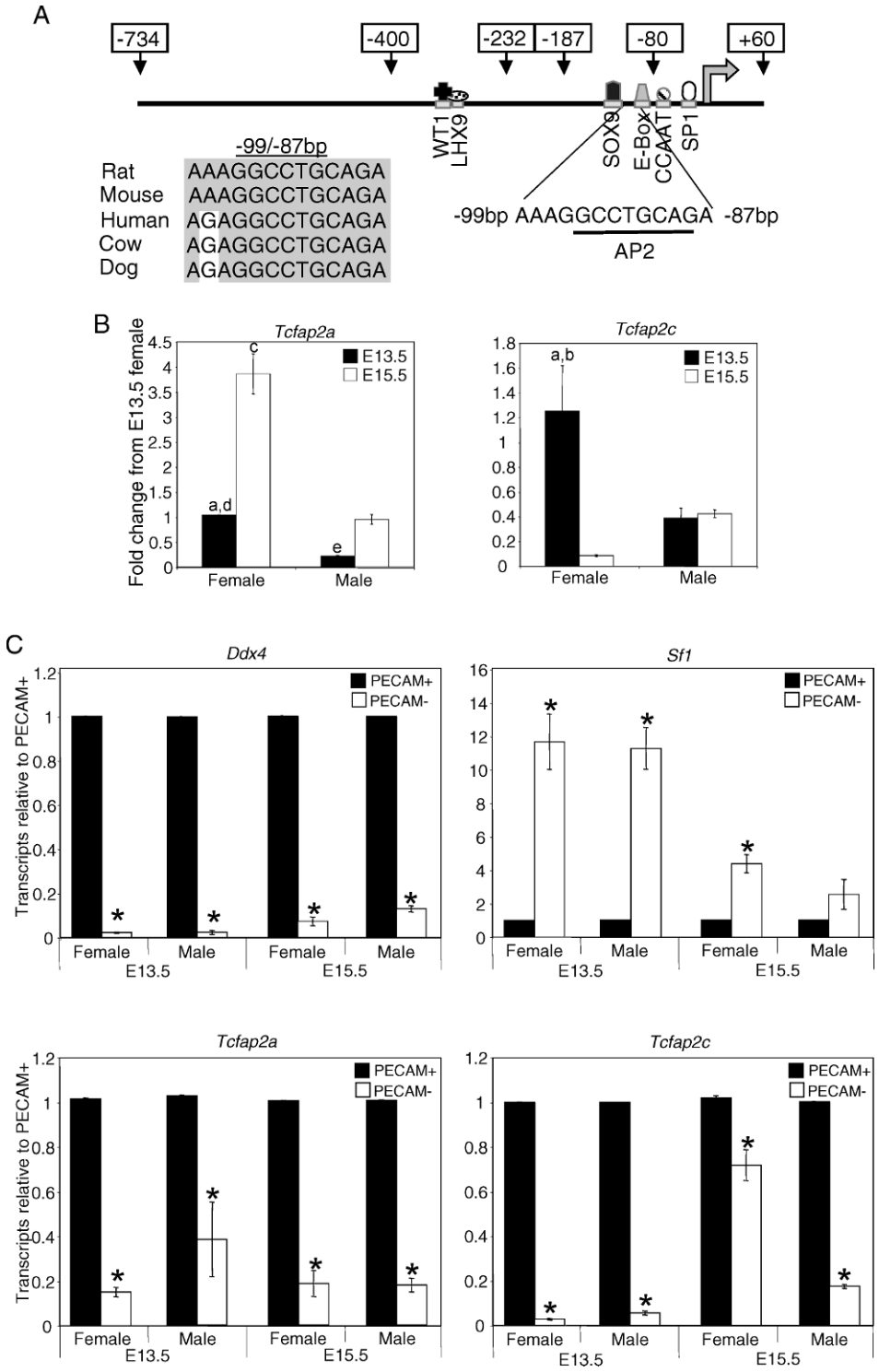


FIG. 6. TCFAP2 factors are expressed in developing gonads. **A**) Relative position of the TCFAP2A/C (AP2α/γ) binding site (−99 to −87 bp) in the context of the −734/+60 bp *Sf1* promoter. Putative TCFAP2A/C-binding sequences are underlined. Conservation of sequences −99/−87 bp is shown between rat (base sequence), mouse, human, cow, and dog. Gray sequences are conserved, and the white background highlights bases that differ from the rat. **B**) QPCR representing average transcript levels of *Tcfap2a* and *Tcfap2c* over time relative to the female gonad at E13.5. Error bars represent the SEM (two-way ANOVA, a: $P < 0.001$ difference between time points within female gonads; b: $P < 0.01$ difference between sex at E13.5; c: $P < 0.001$ difference between sex at E15.5; d: $P = 0.06$ difference between sex at E13.5; e: $P = 0.06$ difference between time points within male gonads). **C**) Male and female gonads were collected at E13.5 and E15.5 and subject to magnetic cell sorting based on the presence of surface antigen, PECAM, a germ and endothelial cell marker. Data shows QPCR results for *Ddx4 (Vasa)*, *Sf1*, *Tcfap2a,* and *Tcfap2c* comparing transcript levels in PECAM− cells relative to PECAM+ cells for each condition (*minimum $P < 0.02$).

To gain information regarding which cell types express TCFAP2 factors, we isolated populations of cells from male and female gonads based on expression of PECAM1, which is a marker of germ and endothelial cells. Samples were isolated from male and female gonads at two distinct time points, E13.5 and E15.5. RNA was harvested from cells isolated into PECAM+ or PECAM− populations by magnetic cell sorting and then processed for QPCR. Transcripts of each gene were assessed in each sex separately as a fold change of PECAM− relative to PECAM+ values at each time point. Transcript levels from PECAM− cells of germ cell marker *Ddx4* (otherwise known as *Vasa*) was barely 2% of PECAM+ (germ cell) expression in both sexes at E13.5 and 10% by E15.5, indicating successful sorting of the cell populations (Fig. 6C). As expected, PECAM− expression of somatic cell marker *Sf1* was over 100-fold higher than PECAM+ transcripts in both sexes at E13.5 and remained substantially higher at E15.5 (Fig. 6C). Like *Ddx4*, *Tcfap2c* expression in PECAM− cells measured at approximately 5% of PECAM+ transcript levels at E13.5; however, at E15.5, PECAM− expression increased, especially in female gonads (Fig. 6C). PECAM− transcript levels of *Tcfap2a* were somewhat higher,

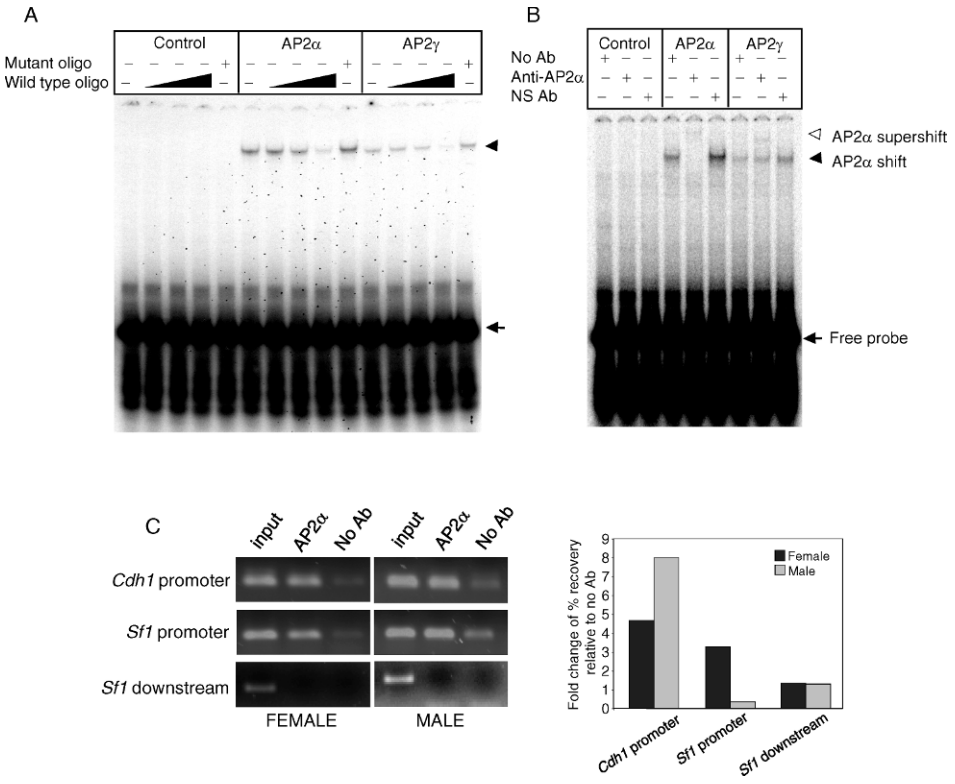


FIG. 7. TCFAP2 factors bind the *Sf1* promoter. **A**) Competition gel mobility-shift assay using labeled oligonucleotides −105 to −77 bp of the *Sf1* promoter together with synthesized negative control (empty vector), TCFAP2A (AP2α), or TCFAP2C (AP2γ) proteins. Unlabeled wild type (1×, 10×, and 100×, represented by increasing density of the ramp) or mutant (100×) oligonucleotides were used to compete with the labeled probe (see *Materials and Methods*). The specific DNA-protein complex is indicated by an arrowhead and the free probe by an arrow. **B**) Supershift assay using labeled probe with synthesized negative control, TCFAP2A or TCFAP2C proteins along with an anti-TCFAP2A (AP2α) or nonspecific (NS) antibody. The DNA-protein complex is indicated by a black arrowhead, the supershifted complex by an open arrowhead, and the free probe by an arrow. **C**) Formaldehyde-cross-linked chromatin from E13.5 gonads was immunoprecipitated with TCFAP2A (AP2α) antibody and regions of *Sf1* chromatin were amplified to show specific binding to the proximal promoter. Representative examples are shown using standard PCR evaluated on a 1% agarose gel (left panel) and QPCR with the values expressed as the fold change of percent recovery with TCFAP2A (AP2α) antibody relative to percent recovery of the no antibody control (right panel). Dark bars, female; light bars, male. Primers were designed to detect sequences within the *Cdh1* promoter (positive control), *Sf1* promoter, and downstream sequences of *Sf1* (nonspecific sequences, negative control).

with levels ranging between 15% and 40% of the expression in PECAM+ cells (Fig. 6C). Finally, we noticed a change in the differences in transcript measurements between PECAM+ and PECAM− populations for *Ddx4* and *Sf1* at E15.5 compared to E13.5 values. These findings were attributed to the relative decrease in PECAM+ cells at E15.5 that is a consequence of rapidly dividing somatic cells, especially in male gonads, during development. Similar data was obtained when transcripts were assessed within germ cell-depleted gonads (Supplemental Fig. S1; all supplemental data are available online at www.biolreprod.org). Based on these data, we conclude that *Tcfap2c* is expressed primarily in germ cells. The striking increase in PECAM− transcript levels in the E15.5 female could at least be partly attributed to extremely low expression in general at that time (Fig. 6B). In addition, our data suggests that *Tcfap2a* expression is present in both germ and somatic cells.

To determine whether TCFAP2A/C could bind *Sf1* promoter sequences −99/−87 bp, we incubated radiolabeled wild type oligonucleotides with synthesized TCFAP2A, TCFAP2C, or negative control in the presence or absence of unlabeled wild type or mutant oligonucleotides. There was no evidence of a migrated band in the negative control samples; however, a single band was detected when the wild type oligonucleotide was incubated with TCFAP2A and TCFAP2C proteins. These bands disappeared in the presence of increasing concentrations of unlabeled wild type oligonucleotides but remained when incubated with excess unlabeled mutant oligonucleotides (Fig. 7A). The addition of an anti-TCFAP2A antibody caused a small fraction of the TCFAP2A-DNA complex to supershift, but the antibody mostly disrupted the complex, causing the band to vanish (Fig. 7B). The TCFAP2A antibody caused a slight supershift of the TCFAP2C-DNA complex, suggesting cross-reactivity. Together, these findings strongly suggest that TCFAP2 factors can bind specifically to −99/−87 bp sequences of the *Sf1* proximal promoter.

We next investigated whether TCFAP2A could bind endogenous *Sf1* sequences in the context of the developing gonad. We performed ChIP analysis by incubating the anti-TCFAP2A antibody with cross-linked chromatin derived from female and male embryonic gonads at E13.5. TCFAP2A antibodies enriched sequences in the proximal promoter of *Cdh1* as expected based on previously reported ChIP results (Fig. 7C) [39]. ChIP results for *Sf1* sequences showed that anti-TCFAP2A associated substantially more with sequences from the female gonad in the proximal promoter of *Sf1* than with sequences that lack TCFAP2 consensus sequences located downstream (Fig. 7C). PCR performed on ChIPs from male gonads resulted in nonspecific or no amplification of the *Sf1* promoter sequence, likely because expression of TCFAP2 is

very low in the male gonads (Fig. 6B). ChIP assays using anti-TCFAP2A were attempted at E15.5 with no positive results (data not shown). Together, the gel shift and ChIP binding assays indicate that TCFAP2 factors can bind to the proximal promoter of *Sf1* in female gonads at E13.5.

DISCUSSION

Transient Transfections into Developing Gonads

Dynamic regulation of genes within specific cells of fetal gonads has not been addressed because there are no appropriate cell lines that represent developing fetal Leydig or Sertoli cells in the male gonad or somatic cells in the female gonad. To overcome this barrier, we developed a technique to assess reporter activity within explant gonad cultures based on a previously reported method [34]. We achieved ample penetration of transfected plasmids into embryonic gonads using the injection and electroporation technique. To validate the procedure, we took advantage of the ubiquitous activity of the early immediate CMV enhancer and localized EGFP activity to germ and somatic cell compartments of gonads. In addition, we successfully used the established dual reporter assay system to quantify reporter activity.

In this study, we chose to analyze the −734/+60 bp *Sf1* promoter, which includes sequences sufficient for *Sf1* expression in the bipotential gonad [30]. This promoter was sufficient to drive reporter activity to appropriate SF1-expressing cells in embryonic gonads of both males and females. Expression could also be differentiated between cell types in the developing gonad. Endogenous *Sf1* expression is substantially lower in female gonads by E15.5; therefore, we tested for temporal changes in reporter activity by transfecting the −734/+60 bp *Sf1* promoter in female and male gonads at this time. No difference in reporter activity was detected between E13.5 and E15.5, suggesting that the minimal *Sf1* promoter harbors insufficient information to regulate time-specific changes and that additional sequences or other epigenetic modifications of the *Sf1* locus were required. Once we validated the integrity of the transfection method, we investigated several mutated *Sf1* promoter constructs and discovered sex-specific elements that contribute to activity. These are discussed in detail below. Cell-specific enhancers have been identified in other studies; however, they are found at significant distances from the *Sf1*-minimal promoter, suggesting a high level of complexity of *Sf1* regulation [31–33]. Thus, analysis of large sequences with this transfection paradigm in parallel to the development of transgenic mice would likely aid in these studies. To that end, we have achieved and are currently optimizing conditions for transfection of BAC (bacterial artificial chromosome) DNA into gonad explant cultures (Kim and Jorgensen, unpublished results). These tools are especially important for future attempts to understand regulation during testis maturation because *Sf1* expression in fetal Leydig cells exceeds that of Sertoli cells over time [40]. Together, these findings verified that the transient transfection technique allows us to reliably quantify promoter activity and monitor cell-specific regulation within the context of the developing gonad.

E-Box but Not SOX9-Binding Sites Regulate the Sf1 *Promoter*

The E-box has been shown to be essential for *Sf1* promoter activity in several studies [23–26]; therefore, it was no surprise that we found abrogated reporter activity in both male and female gonads transfected with the −734/+60 bp construct harboring a mutated E-box sequence. We anticipated that a mutated SOX9 site would affect activity in male gonads because of the specific localization of SOX9 to Sertoli cells. Instead, no difference in reporter activity was detected in gonads of either sex. Although it was possible that reporter activity from Leydig cells masked a decrease in activity from Sertoli cells, the mutated promoter targeted EGFP reporter activity to both cell types. Previously, studies showed that SOX3, SOX8, or SOX9 could bind this site but that SOX9 was solely responsible for stimulating reporter activity in transient transfection assays [29]. These experiments were carried out in HEK293 cells that lack SF1 and may be deficient in other essential factors necessary to assess true *Sf1* promoter-driven reporter activity. This experimental design leaves open the possibility that other SOX factors could bind the promoter in *Sf1*-containing cells [29]. Other explanations for the discrepancy could be that SOX9 is an important factor for Sertoli cell-specific *Sf1* expression but the specific binding sequences are outside the region tested or that SOX9 binding at this site is not important at this particular time in development (i.e., E13.5).

Specific DNA Elements Affect Sf1 *Promoter Activity in Female and Male Gonads*

Two regions within the −734/+60 bp *Sf1* promoter were discovered to contribute to reporter activity in embryonic gonads, +30/+49 bp and −114/−87 bp. The first, +30/+49 bp, exhibited decreased activity only in female gonads when mutated. Sequences including +30/+49 bp are conserved across species, with the human sequence diverging most from rat, mouse, cow, and dog (Supplemental Fig. S2). In silico analysis (TESS) of +30/+49 bp sequences uncovered potential overlapping binding sites for CEBPB/G (C/EBPβ/γ) and ETS2 [41]. QPCR results indicated no difference between male and female gonads in expression of any of these factors; however, immunohistochemistry for CEBPB indicated sexually dimorphic expression at E13.5. Female gonads exhibited CEBPB protein primarily in germ cells, whereas expression in male gonads was colocalized to somatic cells (Supplemental Fig. S2). Ultimately however, these factors did not bind to *Sf1* sequences as determined by binding assays. Thus, the identity of potential factors that bind +30/+49 bp on the *Sf1* promoter remains to be determined.

Examination of 5′-deletion constructs identified sequences between −114 and −87 bp of the *Sf1* promoter as important for reporter activity only in female gonads. To carefully assess this region, six base pair block mutations were made spanning −112 to −87 bp within the context of the −734/+60 bp *Sf1* promoter. To our surprise, μ99 and μ93 constructs exhibited decreased reporter activity in both female and male gonads. This result was obtained when transfections were performed on gonads harvested at E13.5 and E15.5 (data not shown). To determine whether these mutants carried inherent inhibitory activity, we transfected an unrelated SF1-expressing cell line, αT3–1 gonadotrophs. In these cells, the activity of each block mutation was no different from the parent −734/+60 bp *Sf1* promoter construct, suggesting that the decreased activity of μ99 and μ93 was specific to the developing gonads.

TCFAP2 Binds to the Sf1 *Promoter*

Homo- and heterodimers of TCFAP2A and TCFAP2C have been reported to bind variations of a consensus palindromic core sequence, 5′-GCCN$_{3-4}$GGC-3′ [42]. In silico analysis indicated that these factors may bind *Sf1* sequences between −99/−87 bp. Analysis of *Tcfap2a* transcripts suggested that some *Tfap2a* is expressed in somatic cells of developing

gonads. In addition, we discovered that TCFAP2 binds *Sf1* promoter sequences both in vitro and in developing female gonads.

The TCFAP2 family of transcription factors includes five members, A, B, C, D, and E (α, β, γ, δ, and ε), each shown to contribute to specific developmental events [43–52]. *Tcfap2a* knockout and chimeric mice uncovered contributions of TCFAP2A to the development of several independent structures of the embryo. *Tcfap2a*$^{-/-}$ mice harbored notable abnormalities including severe defects in the closure of the cranial neural tube and ventral body wall [44, 45, 53]. Deletion of *Tcfap2c* disrupted formation of extraembryonic tissues and caused early embryonic death [49, 50]. Additional chimeric studies demonstrated that TCFAP2C does not contribute to development of the embryo proper; instead, defects were localized specifically to development and maintenance of extraembryonic tissues [49]. Additional investigations are necessary to determine the functional importance of TCFAP2 factors within the developing gonads.

TCFAP2A has been shown to interact with factors that bind SP1 and E-box sequences to cause both activation and repression of different promoters [54–57]. The TCFAP2-binding site (−99/−87 bp) is immediately adjacent to the E-box and just proximal to Sp1 sequences in the *Sf1* promoter. Thus, it is possible that a regulatory complex is associated with several sequences of the proximal *Sf1* promoter to affect transcription. In the developing female gonad, transfection analysis of −99/−87 and +30/+49 bp regions in the *Sf1* promoter indicate that these regions might interact with each other.

Potential Mechanisms for a Transition to Sexually Dimorphic Regulation of Sf1 *During Gonad Development*

Sf1 expression declines in the developing ovary beginning after E13.5 and remains undetectable until birth. We suppose that there are several potential mechanisms that may contribute to a transition from bipotential to sex-specific regulation of *Sf1* during gonad development. One mechanism may rely on changes in expression of factors that are initially required for *Sf1* regulation in both male and female gonads during the bipotential stage of development, but diverge during sex differentiation. Alternatively, regulatory elements within the *Sf1* locus may be dynamic, and their requirements change in importance over time. The transient transfection data convincingly demonstrates that sequences within the proximal promoter of *Sf1* affect activity in female gonads at E13.5. This result suggests that the requirements of the elements can be truly sexually dimorphic no matter what factors they bind.

Several kilobases of *Sf1* sequences have been used in attempts to identify enhancer regions that drive *Sf1* expression to specific tissues. Recent studies have also highlighted the potential importance of epigenetic modifications in tissue-specific expression and show that DNA methylation also contributes to cell-specific regulation of *Sf1* [58]. Certainly these additions along with specific marks on the chromatin will likely contribute to temporal and spatial regulation of the *Sf1* gene. Ultimately, we anticipate that the decline of *Sf1* expression in developing ovaries is caused by complex mechanisms that likely include changing requirements of regulatory elements, limited access to specific transactivating factors, binding of repressor factors, and epigenetic events. Future studies will focus on investigations into the molecular machinery that enhances or represses *Sf1* expression in specific cell types during gonad development. Here, we highlight a novel technique used to uncover potential transcription factor-binding sites that contribute to sex-specific and cell-specific regulation of *Sf1* during a specific window in time in developing gonads.

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