

USF1/2 Transcription Factor DNA-Binding Activity Is Induced During Rat Sertoli Cell Differentiation¹

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ABSTRACT

Each Sertoli cell can support a finite number of developing germ cells. During development of the testis, the cessation of Sertoli cell proliferation and the onset of differentiation determine the final number of Sertoli cells and, hence, the number of sperm that can be produced. We hypothesize that the transition from proliferation to differentiation is facilitated by E-box transcription factors that induce the expression of differentiation-promoting genes. The relative activities of E-box proteins were studied in primary Sertoli cells isolated from 5-, 11-, and 20-day-old rats, representing proliferating, differentiating, and differentiated cells, respectively. E-box DNA-binding activity is almost undetectable 5 days after birth but peaks with initiation of differentiation 11 days after birth and remains elevated. Upstream stimulatory factors 1 and 2 (USF1 and USF2) were found to be the predominant E-box proteins present within DNA-protein complexes formed after incubating E-box-containing probes with nuclear extracts from developing Sertoli cells. The known potentiator of Sertoli cell differentiation, thyroxine, increases USF DNA-binding activity in Sertoli cells before differentiation (5-day-old Sertoli cells) but not after differentiation is initiated (11- and 20-day-old Sertoli cells). The developmental-specific increase in USF1 and USF2 DNA-binding activity may facilitate the switch from proliferation to differentiation and, thus, determine the ultimate number of Sertoli cells present within the testes and the upper limit of fertility.

bHLH proteins, E-box factors, follicle-stimulating hormone, follicle-stimulating hormone receptor, gene regulation, ID proteins, spermatogenesis, testis

INTRODUCTION

Sertoli cells are required to support male germ cell development (spermatogenesis) in the mammalian testis. Sertoli cells are capable of supporting only a finite number of germ cells [1]. Therefore, the final number of Sertoli cells sets the upper limit for testicular sperm production and determines the level of male fertility [2, 3]. Sertoli cells do not divide after puberty, and apoptosis of these cells is rare; therefore, the number of Sertoli cells is primarily determined by the expansion of Sertoli cells before the completion of puberty [4, 5]. Sertoli cell proliferation and the decision to

differentiate are regulated by a number of hormonal cues, including thyroid hormone, retinoic acid, activin, FSH, and testosterone [6]. The regulation of Sertoli cell development is also dependent on cell-cell interactions, signals from the extracellular matrix, and factors from developing germ cells [6]. Although numerous factors regulating Sertoli cell development have been identified, little is known about the molecular mechanisms in Sertoli cells that halt cell division and trigger differentiation.

During the period in which proliferation decreases, the Sertoli cell undergoes a developmental process that results in a fully differentiated, nonproliferating cell that is capable of supporting spermatogenesis. The decrease in Sertoli cell proliferation during the differentiation period has been well documented in rats [4, 7, 8] and mice [9]. The percentage of thymidine-labeled Sertoli cells is 33% at birth and 32% at 5 days after birth but decreases to 18% and 7% at 10 and 11 days, respectively. By 16 days after birth, labeled Sertoli cells can no longer be detected [10].

The Sertoli cell differentiation process includes morphological changes, the induction of secreted proteins that are required by germ cells, and the formation of specialized tight junctions between Sertoli cells that establish the blood-testis barrier [6]. Potential mediators of Sertoli cell differentiation signals include members of the basic helix-loop-helix (bHLH) family of transcription factors. The bHLH transcription factors previously shown to be expressed in Sertoli cells include scleraxis (*Scx*), upstream stimulatory factors 1 and 2 (*Usf1* and *Usf2*), REB α isoform (the rat homologue of the human HeLa E-box-binding protein, HEB [*Tcf12*]), and E47 but not E12 proteins that are derived from the *Tcfe2a* gene (formerly known as *E2a*) [11–13]. The bHLH transcription factors bind to specific DNA sequence motifs known as E-boxes (CANNTG), closely related N-boxes (CACNAG), and Ets-binding sites (GGAA/T) [14–16]. In general, two categories of proteins bind to E-boxes based on the sequence of the binding motif. E-boxes having the sequence CAGCTG bind E47, E12, and REB α , whereas E-boxes having the sequence CACGTG bind USF proteins and MYC [17]. E-box motifs are known to regulate the promoters of a number of Sertoli cell-specific genes that are markers for differentiated status, including transferrin (*Trf*), steroidogenic factor 1 (*Nr5a1*), and the follicle-stimulating hormone receptor (*Fshr*) [18–22].

E-box protein actions can be inhibited by repressor proteins called inhibitors of cell differentiation or inhibitors of DNA binding (ID). Four ID proteins have been characterized in mammalian cells, ID1, ID2, ID3, and ID4 [23–26]. All four ID proteins contain a conserved helix-loop-helix (HLH) dimerization motif that mediates interactions with other HLH proteins [14, 16]. However, these proteins lack the basic DNA-binding domain and function as dominant negative repressors of bHLH transcription factors by forming non-DNA-binding, inactive heterodimers. A major consequence of ID protein expression is the inhibition of bHLH protein-mediated

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TABLE 1. Electrophoretic mobility shift assay probes.

DNA motif	Template
USF consensus E-box	5'-CAC CCG GTC ACG TGG CCT ACA-3'
<i>Fshr</i> promoter E-box	5'-GAT CCG TGG GTC ACG TGA CTT TGC-3'
E47 consensus E-box	5'-AGC TTC CCA GCT GAG GAA TTC CAC AGC TGG GC-3'
<i>Trf</i> promoter E-box	5'-GAT CGC CCA AGC AGC TGT ACC ATG C-3'
<i>Id2</i> promoter E-box	5'-CGG GGC GCG AGC CCA GCT GGG GTG GTA AAT A-3'
CREB consensus CRE	5'-GAT CCG GCT GAC GTC ATC AAG CTA GAA-3'

activation of genes required for the differentiation of tissues during development [27]. ID proteins have been shown to repress the activity of at least two E-box protein-activated genes in Sertoli cells, namely, *Trf* and *Fshr* [18, 20].

We tested the hypothesis that E-box protein activity and E-box-mediated transcription are regulated during the transition from proliferation to differentiation within Sertoli cells. We examined E-box DNA-binding activity and the major E-box proteins responsible for DNA-protein interactions within Sertoli cell nuclei from 5-, 11-, and 20-day-old Sertoli cells, corresponding to proliferating, differentiating, and differentiated Sertoli cells, respectively. The levels of E-box protein expression and E-box-mediated transcription were assessed. Finally, the effects of thyroid hormone, a promoter of differentiation, on E-box protein DNA-binding ability and on ID and E-box mRNA expression were evaluated.

MATERIALS AND METHODS

Animal Care and Use

Male Sprague-Dawley rat pups were obtained from Charles River Laboratories (Boston, MA). Animals used in these studies were maintained and euthanized according to the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Isolation of Sertoli Cells for Direct Assay

Rats were euthanized 5 and 11 days after birth, and Sertoli cells were isolated as described by Anway et al. [28], with slight modifications. Briefly, decapsulated testes were digested with collagenase (0.5 mg/ml, 34°C, 10–15 min, 80 oscillations/min) in enriched Krebs-Ringer bicarbonate medium (EKRB [118.5 mM NaCl, 4.7 mM KCl, 2.1 mM CaCl₂-H₂O, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄-7H₂O, 25 mM NaHCO₃, 10 mM Hepes, and 0.1% BSA]), followed by three settlings in EKRB to isolate seminiferous tubules. Tubules were dispersed through digestion with trypsin (0.5 mg/ml) in the presence of DNase (1 µg/ml) for 5–10 min at 37°C without shaking. Following the digestion, tubule fragments were washed with soybean trypsin inhibitor (0.3 mg/ml), followed by two washes with EKRB. Sertoli cells were separated from germ cells by incubation with 0.1% collagenase, 0.2% hyuronidase, 0.04% DNase I, and 0.03% trypsin inhibitor (40 min, 34°C, 80 oscillations/min). Following digestion, cells were pelleted and washed three times with EKRB. To remove contaminating germ cells, the suspension of single cells and clusters of 5–10 cells was subjected to hypotonic shock by resuspending the pellet in EKRB diluted with water (final concentration, 0.2× EKRB), gently inverting three times to disperse cells, followed by immediate centrifugation at 700 rpm (63 × g) for 10 min. Cells were then washed three times with serum-free media containing 50% Dulbecco modified Eagle medium (DMEM), 50% Ham F-12, 5 mg/ml of insulin, 5 mg/ml of transferrin, 10 ng/ml of epidermal growth factor, 3 µg/ml of cytosine β-D-arabino furanoside, 1 mM sodium pyruvate, 200 U/ml of penicillin, and 200 µg/ml of streptomycin. The resuspended cells were counted and directly aliquoted for preparation of protein extracts or plated for analysis of purity.

Isolation of Sertoli Cells for Cell Culture

Rats were euthanized 5, 11, and 20 days after birth as described previously [29]. Decapsulated testes were digested with collagenase (0.5 mg/ml, 33°C, 12 min) in EKRB, followed by three settlings in EKRB to isolate seminiferous

tubules. The tubules were digested with trypsin (0.5 mg/ml, 32°C, 12 min) in the presence of DNase (2 µg/ml), and cell aggregates were passed repeatedly through a Pasteur pipette. An equal volume of DMEM containing 10% fetal bovine serum was added to the Sertoli cells, which were then pelleted (500 × g, 5 min) and resuspended in supplemented serum-free media. Sertoli cells were cultured on dishes coated with Matrigel (Collaborative Research, Bedford, MA) (33°C, 5% CO₂). Sertoli cells were routinely 95% pure as determined by phase microscopy and alkaline phosphatase staining [30]. Cells were cultured 3 days before treatment. Sertoli cells were stimulated with vehicle (ethyl alcohol) or thyroxine (T4 [10⁻⁶ M]) for 24 h.

Determining Purity of Sertoli Cells

In a 24-well plate, 12-mm² glass coverslips were coated with poly-L-lysine and allowed to dry. Coverslips were washed with PBS, and 2.5 × 10⁴ cells were plated per well. Cells were allowed to attach to coverslips for approximately 2 h, after which Sertoli cells and peritubular cells were identified by immunofluorescence. Briefly, coverslips were washed with PBS and fixed with 4% formalin for 15 min at room temperature and stored in 70% ethanol overnight. The coverslips were stained with antisera against Sertoli cell-specific vimentin (V6630; Sigma-Aldrich, St. Louis, MO) or with antisera against peritubular cell-specific alpha smooth muscle actin (A2547; Sigma-Aldrich). Primary antisera were detected by secondary antibodies tagged with Alexa 488 or Cy5. Nuclei were visualized using Hoechst 33258 (861405; Sigma-Aldrich). Leydig cells were detected by staining with the lipid-specific stain Oil Red O (O0625; Sigma-Aldrich). The cells were fixed in 2% paraformaldehyde for 5 min, washed three times with distilled water, incubated in propylene glycol for 5 min, and stained with 0.5% Oil Red O in propylene glycol for 8 min at 60°C. Cells were rinsed in 85% propylene glycol for 5 min and washed with distilled water before staining with hematoxylin. Cells were washed repeatedly with water during 3 min, followed by two changes of distilled water before mounting. All images were taken on an Olympus Provis AX70 Microscope (Olympus, Tokyo, Japan), and quantification was performed using Northern Eclipse v. 7.0 Software (MVA Inc., Monaca, PA). Ten random fields from multiple coverslips were analyzed to determine the total number of nuclei and the number of cells expressing each marker. The mean (±SEM) percentage of cells expressing the cell-specific marker was calculated. More than 500 cells were counted for each detection method.

Nuclear Protein Extracts

Freshly isolated Sertoli cells (1.5×10⁶ cells/sample) or three 100-mm² plates of cultured Sertoli cells were collected in a total of 1 ml of 1× PBS and used for the preparation of nuclear extracts. Nuclear and cytoplasmic extracts were prepared by detergent lysis [31]. Briefly, after pelleting, the cells were incubated in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, and a protease inhibitor cocktail) for 15 min on ice, followed by the addition of 0.06% Nonidet P40 (Sigma-Aldrich). Cells were vortexed for 10 sec, and nuclei were collected by centrifugation (12 000 × g, 30 sec). The nuclei pellet was then washed once with buffer A and resuspended in buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). Nuclei were incubated for 15 min at 4°C with shaking. The cell debris was pelleted (12 000 × g, 5 min), and the supernatant containing nuclear proteins was stored at -80°C. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay; Bio-Rad Laboratories, Inc., Hercules, CA).

DNA-Protein Binding Studies

Radiolabeled DNA probes were generated by annealing nucleotide templates containing protein-binding motifs plus flanking promoter sequences to 10-nucleotide primers that are complementary to the 5' end of the templates (Table 1). The overhangs were filled in with Klenow enzyme (Promega,

TABLE 2. Primers for qPCR.

Gene	Accession no.	Sequence	Region amplified	Reference ^a
<i>Usf1</i>	NM_031777	Forward 5'-AAGTCAGAGGCTCCAGGA-3' Reverse 5'-CGGCGCTCCACTTCGTTAT-3'	693–761	[36]
<i>Usf2</i>	NM_031139	Forward 5'-AGACCAACCAGCGTATGCAG-3' Reverse 5'-GTCCTCGGATCTGCTGCC-3'	1386–1473	[36]
<i>Tcfe2a</i> (E47)	NM_01107865	Forward 5'-CACCTTCGGGAGAGACACCA-3' Reverse 5'-AGGAAGCCAGCCTGACTCAG-3'	304–354	
<i>Id1</i>	NM_012797	Forward 5'-CGACTACATCAGGGACCTGCA-3' Reverse 5'-GAACACATGCCGCCCTCGG-3'	336–479	[37]
<i>Id2</i>	NM_013060	Forward 5'-ATCCCCAGAACAAGAAGGT-3' Reverse 5'-CTGTCCAGGTCTCTGGTGGT-3'	220–349	[38]
<i>Id3</i>	NM_013058	Forward 5'-GCATCTCCCGATCCAGACAG-3' Reverse 5'-TTGGAGATCACAGTTCCGGA-3'	299–349	
<i>Id4</i>	NM_175582	Forward 5'-CGATAACGTGCTGCAGGATCT-3' Reverse 5'-AGGCTCGTGCCACCAT-3'	229–295	[39]
<i>Trf</i>	NM_001013110	Forward 5'-CTGTGACCTGTGTATTGGCC-3' Reverse 5'-CGTCTCCCTTCTCAACGAGG-3'	1575–1675	
<i>Ppia</i>	M19533.1	Forward 5'-ATGGTCAACCCACCGTGT-3' Reverse 5'-TCTGTGTCTTTGGAACCTTGTCT-3'	43–143	

^a Primers without a reference were designed for use in this study with the aid of Primer Express Software v 3.0 (Applied Biosystems).

Madison, WI) in the presence of [α -³²P] dATP and 5 mM each of dCTP, dGTP, dTTP and 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdU). Electrophoretic mobility shift assays (EMSA) were performed as described [32]. Briefly, ³²P-labeled probes were incubated with nuclear extracts (0.2–1.0 μ g). Binding reactions were incubated for 15 min at room temperature in the presence of 1 μ g of poly deoxyinosinic-deoxycytidylic acid (dI-dC), 0.25 mg/ml of BSA, 5 mM DTT, 50–100 mM KCl, 20 mM Hepes, pH 7.9, 20% glycerol, and 0.2 mM EDTA. For supershift assays, antiserum was added to the mixture for 15 min at room temperature before the addition of the probe. All of the following antisera were obtained from Santa-Cruz Biotechnologies (Santa Cruz, CA): α -USF1 (sc-229), α -USF2 (sc-862), α -E47 (sc-763), α -E2A.E12 (sc-349), and α -MYC (sc-40). Protein-DNA complexes were resolved via 5% PAGE under non-denaturing conditions in a Tris borate/EDTA buffer. The gels were dried and exposed to Classic X-Ray Film, Blue Sensitive (Laboratory Products Sales, Rochester, NY) at -80°C for 1 h to 1 wk. DNA-protein complex formation was quantified from digitized images of the x-ray films using ImageJ v. 1.37 software [33, 34]. Results from at least three independent experiments were analyzed by ANOVA with Fisher probable least-squares difference at a 5% significance level using Statview 4.5 (Abacus Concepts, Inc., Berkeley, CA).

RNA Isolation and cDNA Preparation

RNA was obtained from Sertoli cells directly isolated from testis tissue using RNA STAT60 (Tel-Test, Friendswood, TX) and from cultured Sertoli cells using RNeasy Mini Kit (Quiagen, Inc., Valencia, CA). After digestion with RNase-free DNase, the RNA was subjected to reverse transcription using random hexamers [35]. For reverse transcription, 250 ng of RNA was incubated with 100 μ l of reaction mix containing 7.5 mM MgCl₂, 400 μ M deoxynucleotide triphosphates (Promega, Madison, WI), 10 \times PCR II Buffer (Applied Biosystems, Foster City, CA), 40 U of RNasin RNase inhibitor (Promega), 2.25 μ M random hexamers (Integrated DNA Technologies, Coralville, IA), 250 U of Superscript RT II (Invitrogen, San Diego, CA), and nuclease-free water (Ambion, Austin, TX). Parallel reactions were performed without RT to control for the presence of contaminant DNA. The samples were incubated at 25 $^{\circ}\text{C}$ for 10 min, at 48 $^{\circ}\text{C}$ for 30 min, and at 95 $^{\circ}\text{C}$ for 5 min, followed by 4 $^{\circ}\text{C}$ for 5 min.

Quantitative PCR Analysis of Gene Expression

Real-time PCR (quantitative PCR [qPCR]) amplifications were performed in a 96-well plate in the ABI Prism 7900HT Sequence Detection System v. 2.3 (Applied Biosystems) in a total volume of 25 μ l, which included 2 μ l of cDNA, 12.5 μ l of Absolute SYBR Green ROX Mix (ThermoFisher Scientific, ABgene House, Surrey, England), and 600 μ M of each primer. Primers used for each gene are listed in Table 2. Previously undescribed primers were designed using Primer Express Software v. 3.0 (Applied Biosystems). All primers were independently validated for use in the $\Delta\Delta\text{Ct}$ method of gene expression analysis [40] through the use of a standard curve derived from serial dilutions of the cDNA obtained from the reverse transcription reactions. The resulting cycle threshold (Ct) values for each sample were plotted vs. the log of the mRNA concentration present in each cDNA dilution. Ct is the cycle number at

which the fluorescent signal of the amplification product exceeds background levels. The slope of the line was used to calculate the efficiency of amplification (Efficiency = $10^{1/(-\text{slope})} - 1$). Primers with a mean (\pm SEM) efficiency of 1 ± 0.1 were considered acceptable. *Ppia* (peptidylprolyl isomerase A, commonly known as cyclophilin) was used as an endogenous control. The qPCR analysis was initiated with melting of cDNA at 95 $^{\circ}\text{C}$ for 15 min, followed by 40 amplification cycles (15 sec at 95 $^{\circ}\text{C}$ and 1 min at 60 $^{\circ}\text{C}$). A dissociation curve was performed immediately after amplification to ensure that there was only one (gene specific) amplification peak.

Ct values were recorded and analyzed via the $\Delta\Delta\text{Ct}$ method and via the efficiency-corrected ΔCt method [40]. The $\Delta\Delta\text{Ct}$ method was used to characterize relative fold changes in mRNA expression between treatment groups. The following terms were defined before calculation: GOI indicates gene of interest; reference, Ct value for GOI of 5-day-old untreated sample; unknown, Ct value for GOI of samples from any time point or treatment; and control, Ct value of *Ppia* for a given treatment. The ΔCt was calculated for each sample according to the following formula: $\Delta\text{Ct} = \text{Unknown} - \text{Control}$. The $\Delta\Delta\text{Ct}$ was calculated by comparing each sample with the reference according to the following equation: $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{unknown}} - \Delta\text{Ct}_{\text{reference}}$. The fold change was then calculated relative to the reference according to the following formula: $\text{Fold Change} = 2^{(-\Delta\Delta\text{Ct})}$. The means (\pm SEMs) of 3 individual experiments were determined for each GOI in each treatment group. The relative quantity of mRNA for each GOI was determined by the efficiency-corrected ΔCt method. The relative quantity is derived from the following equation: $\text{Quantity} = (\text{Efficiency} + 1)^{-\text{Ct}}$. For each sample, the calculated quantity is then normalized to the quantity found for cyclophilin. The means (\pm SEMs) of 3 individual experiments were determined for each treatment group for each gene of interest. Results were analyzed by ANOVA with Fisher probable least-squares difference at a 5% significance level using Statview 4.5.

RESULTS

Isolation of Highly Enriched Fractions of Proliferating and Differentiating Sertoli Cells for Immediate Biochemical Analysis

To test the hypothesis that E-box transcription factor activities are up-regulated during the development of the Sertoli cell, the DNA-binding activity of E-box proteins was assayed in Sertoli cells isolated from the testes of 5- and 11-day-old rats, representative of proliferating and differentiating Sertoli cells, respectively. To examine Sertoli cell biochemical properties as close to in vivo conditions as possible, enriched fractions of Sertoli cells were assayed immediately after isolation from rat testes using an adaptation of the protocol by Anway and colleagues [28]. Using this protocol, we obtained approximately 1.7×10^7 cells from ten 5-day-old rat pups and 9.0×10^7 cells from ten 11-day-old rat pups. Imaging analysis confirmed that the isolation procedure resulted in

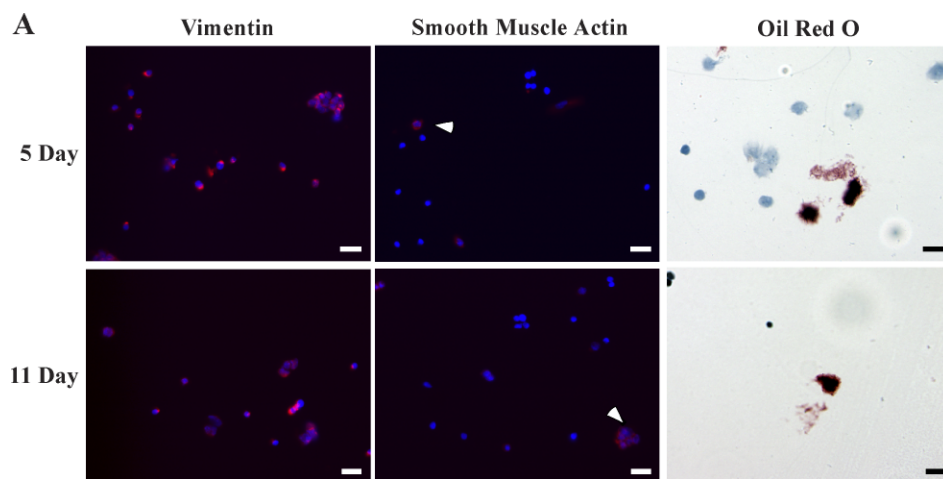
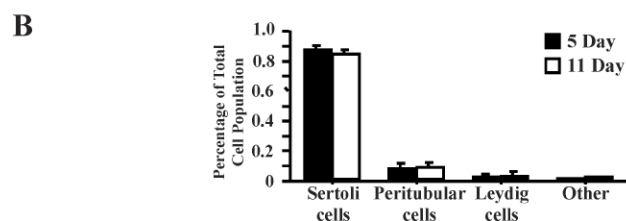


FIG. 1. Enriched fractions of Sertoli cells are obtained immediately after enzymatic digestion of testis tissue. Cell suspensions obtained immediately after enzymatic digestion of rat testes were cultured on coverslips for 2 h and then fixed. **A**) Sertoli cells were detected using antisera against vimentin (red, left column), peritubular cells were detected by antisera against alpha smooth muscle actin (red, white arrows, middle column), and nuclei were stained with Hoechst dye (blue). Leydig cells were stained with the lipid stain Oil Red O (red stain, right column). Bar = 20 μ m. **B**) Quantitation of the mean (\pm SEM) percentage of cell-specific markers of 10 random fields from multiple coverslips is provided.



highly enriched Sertoli cell fractions (Fig. 1A). Approximately 85% of the cells stained with the Sertoli cell-specific marker vimentin, whereas approximately 9% stained with the peritubular cell marker alpha smooth muscle actin, and 3% to 9% stained positive for the Leydig cell marker Oil Red O (Fig. 1B). One percent of cells were not stained and are most likely immature germ cells.

DNA-Binding Activities of USF Proteins Increase During Sertoli Cell Differentiation

EMSAs were performed with nuclear extracts from the freshly isolated Sertoli cells. The extracts were incubated with

radiolabeled, double-stranded oligonucleotide probes containing E-box motifs that are selective for the binding of specific E-box proteins. For probes containing a consensus USF-binding site or a region of the *Fshr* promoter containing an E-box known to bind USF [13, 41], protein binding was low or undetectable in extracts from 5-day-old Sertoli cells, but binding activity increased at least 2-fold at 11 days (Fig. 2A). As expected, CREB1 binding to a consensus CREB-binding motif, which is generally stable under most conditions, was unchanged at 5 and 11 days [42].

To identify E-box-binding proteins that are responsible for forming the DNA-protein complexes, supershift studies were

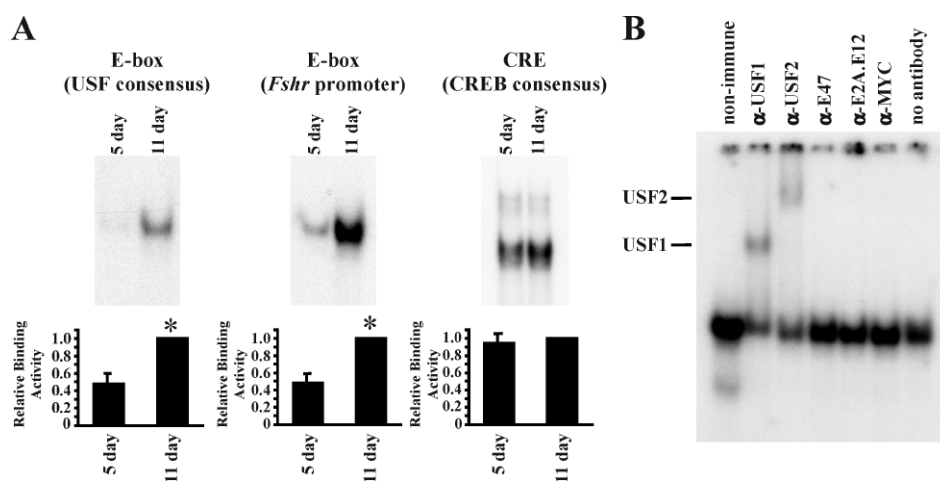
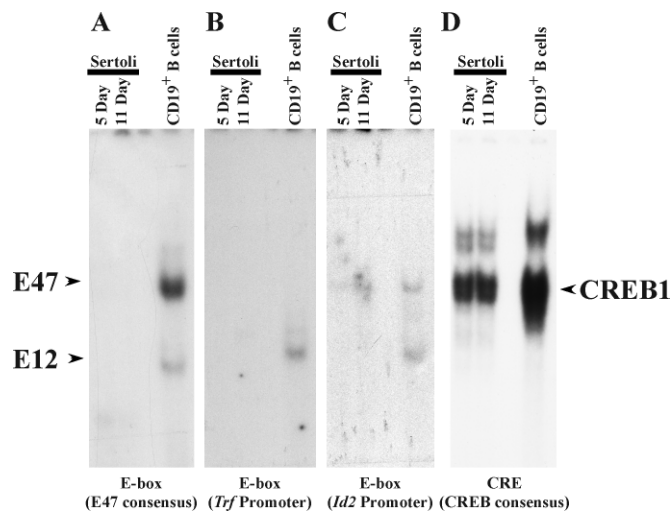


FIG. 2. DNA-binding activity increases during Sertoli cell differentiation. Nuclear extracts isolated from Sertoli cells immediately after enzymatic digestion were assayed for DNA-protein interactions. **A**) In EMSAs, radiolabeled probes containing 1) a consensus E-box that is selective for USF binding, 2) a region of the *Fshr* promoter containing an E-box known to bind USF proteins, or 3) a CREB-binding site (CRE) were incubated with Sertoli cell nuclear extracts isolated from 5- and 11-day-old rats. Representative images of the resulting DNA-protein complexes are shown, and quantitation of the mean (\pm SEM) of five independent experiments is provided. The relative binding activity for each probe is normalized to that of 11-day-old Sertoli cells (\equiv 1). Statistically significant differences ($P < 0.05$) are indicated by an asterisk (*). **B**) Nuclear extracts of enriched Sertoli cells from 11-day-old rats were incubated with nonimmune sera or antisera against USF1, USF2, E47, E2A, or MYC. Supershifted DNA-protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h.



account for increased binding to E-box motifs during Sertoli cell differentiation.

DNA-Binding Activity of the E47 E-box Protein Is Low in 5- and 11-Day-Old Sertoli Cells

The E47 E-box protein has been implicated in numerous activities in Sertoli cells, including FSH-induced regulation of *Trf* [22] and the influencing of hormone-induced activation of the *Fos* promoter through the serum response element [43]. To investigate further the binding activity of E47 and the related E12 E-box factors, nuclear extracts of Sertoli cells isolated from 5- and 11-day-old rats were incubated with probes that were previously shown to bind E47, including a consensus binding site for E47, the *Trf* promoter E-box, or an E-box from the *Id2* promoter region [22, 43, 44]. Incubation of the E47-selective probes with Sertoli cell nuclear extracts did not result in detection of any DNA-protein complexes (Fig. 3, A–C). In contrast, all probes formed complexes with nuclear extracts derived from splenic origin CD19⁺ B cells activated with lipopolysaccharide, which are known to contain high levels of E47- and E12-binding activity [45–47]. Using a probe containing a binding site for the CREB1 transcription factor, the protein-binding activity of the Sertoli nuclear extracts was confirmed by the finding that DNA-protein complexes were formed (Fig. 3D). Together, these studies indicate that E47-binding activity is below detectable limits in the nuclei of 5- and 11-day-old Sertoli cells.

USF DNA-Binding Activity Is Similar in Freshly Isolated and Cultured Sertoli Cell Models

To facilitate studies of factors that may regulate Sertoli cell differentiation and E-box-binding activity, Sertoli cells from 5- and 11-day-old rats were studied after being placed in culture. In addition, fully differentiated Sertoli cells from 20-day-old rats were cultured. By using a gentler isolation procedure and

performed. Sertoli cell nuclear extracts prepared from 11-day-old testes were preincubated with nonimmune sera or antisera against USF1, USF2, E47, E2A, or MYC before incubation with the E-box region of the *Fshr* promoter (Fig. 2B). The supershift assay demonstrated that both USF antisera upshifted or disrupted DNA-protein complexes, whereas the E47, E2A, and MYC antisera had little effect. These data suggest that USF1 and USF2 proteins are the major E-box factors that

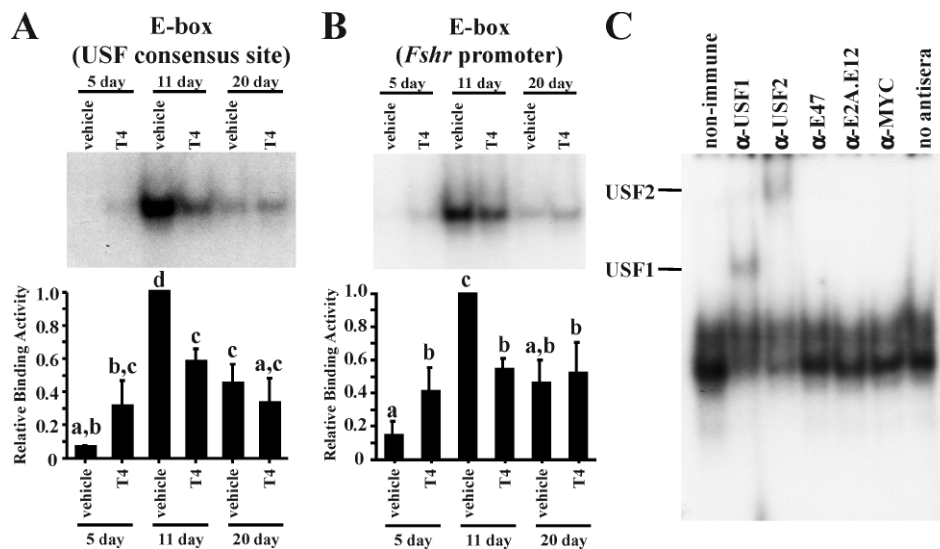


FIG. 4. E-box DNA-binding activity increases during Sertoli cell differentiation. In EMSAs, radiolabeled probes containing a consensus E-box that is selective for USF binding (A) or a region of the *Fshr* promoter containing an E-box known to bind USF proteins (B) were incubated with nuclear extracts isolated from cultured 5-, 11-, and 20-day-old Sertoli cells that were treated with vehicle or T4 (10^{-6} M). Representative images of the resulting DNA-protein complexes are shown, and quantitation of the mean (\pm SEM) of three independent experiments is provided. The relative binding activity for each condition is normalized to that of vehicle-treated 11-day-old Sertoli cells ($\equiv 1$). Values with different lowercase letters differ significantly ($P < 0.05$). C) USF1 and USF2 are responsible for the increase in DNA-binding activity during Sertoli cell differentiation. DNA-protein complexes from nuclear extracts isolated from cultured 11-day-old Sertoli cells were incubated with nonimmune sera or antisera for USF1, USF2, E47, E2A, or MYC. Supershifted DNA-protein complexes containing USF1 or USF2 are indicated. Exposure times for all complexes are less than 48 h.

maintaining the Sertoli cells in culture for 4 days, cell survival was increased compared with the protocol by Anway et al. [28]. Previous investigations have shown that Sertoli cell populations are more than 95% pure 4 days after initiating the cultures [29].

EMSA analysis of nuclear extracts from Sertoli cells cultured from 5- and 11-day-old rats was similar to the analysis using freshly isolated cells in that E-box protein binding to the USF consensus and *Fshr* promoter probe was low at 5 days but increased 5- to 10-fold at 11 days (Fig. 4, A and B). E-box-binding activity decreased at 20 days but was still 4- to 5-fold greater than E-box-binding activity present in 5-day-old Sertoli cells. As shown for freshly isolated Sertoli cells, supershift analysis of the cultured Sertoli cell nuclear extracts indicated that USF1 and USF2 proteins were the predominant E-box factors interacting with the *Fshr* promoter E-box (Fig. 4C). In agreement with the results obtained from freshly isolated Sertoli cells, E47 DNA-binding activity assayed using three E47-selective probes was at or below the level of detection in extracts from cultured 5-, 11-, or 20-day-old Sertoli cells (data not shown). Together, these data confirm that the developmental profile of E-box protein-binding activities and the prevalence of USF-binding activity are similar in freshly isolated and cultured Sertoli cells.

Thyroid Hormone Induces USF DNA-Binding Activity in Proliferating Sertoli Cells

To determine whether thyroid hormone, a known Sertoli cell-differentiating agent, could increase the DNA-binding activity of USF proteins, cultured Sertoli cells were incubated with vehicle or T4 (10^{-6} M) for 24 h. Thyroxine was used because T4 levels are 100-fold greater than triiodothyronine (T3) levels in the testis during the period of Sertoli cell differentiation [48]. Furthermore, Sertoli cells have been shown to express iodothyronine deiodinase type 2, which converts T4 to T3, the more potent form of thyroid hormone [49]. The dose of T4 that was used is 12.5-fold greater than the peak T4 serum level (8×10^{-8} M) reached in rats during differentiation [48]. A 24-h stimulation of cultured Sertoli cells from 5-day-old rats with T4 resulted in induced protein binding to the USF consensus and *Fshr* promoter E-box probes (Fig. 4, A and B). In contrast, T4 stimulation was unable to further induce E-box protein binding in Sertoli cells from 11- and 20-day-old rats. Furthermore, at 11 days, T4 caused a significant reduction in protein binding to the USF consensus and *Fshr* promoter E-box probes. Results of these studies suggest that T4 is able to induce USF DNA-binding activity in Sertoli cells before the commitment to differentiation.

Usf1 mRNA Expression Increases During Sertoli Cell Differentiation

To determine whether increased USF DNA-binding activity during Sertoli cell differentiation corresponded with increased *Usf* expression, the expression of mRNAs encoding E-box proteins was assayed during Sertoli development using qPCR. Assays of mRNA preparations from Sertoli cells immediately after isolation from 5- and 11-day-old rat testes revealed a 62% increase in *Usf1* mRNA levels 11 days after birth. *Usf2* and *Tcfe2a* (E47) mRNA levels also trended higher (24% for both) but did not reach a level of statistical significance (Fig. 5, A–C). *Trf* mRNA levels increased from 5 to 11 days, in agreement with previous studies [50, 51] showing that *Trf* mRNA levels increase during differentiation (Fig. 5D). The mRNA levels of the housekeeping gene *Ppia* did not change during the course of differentiation (data

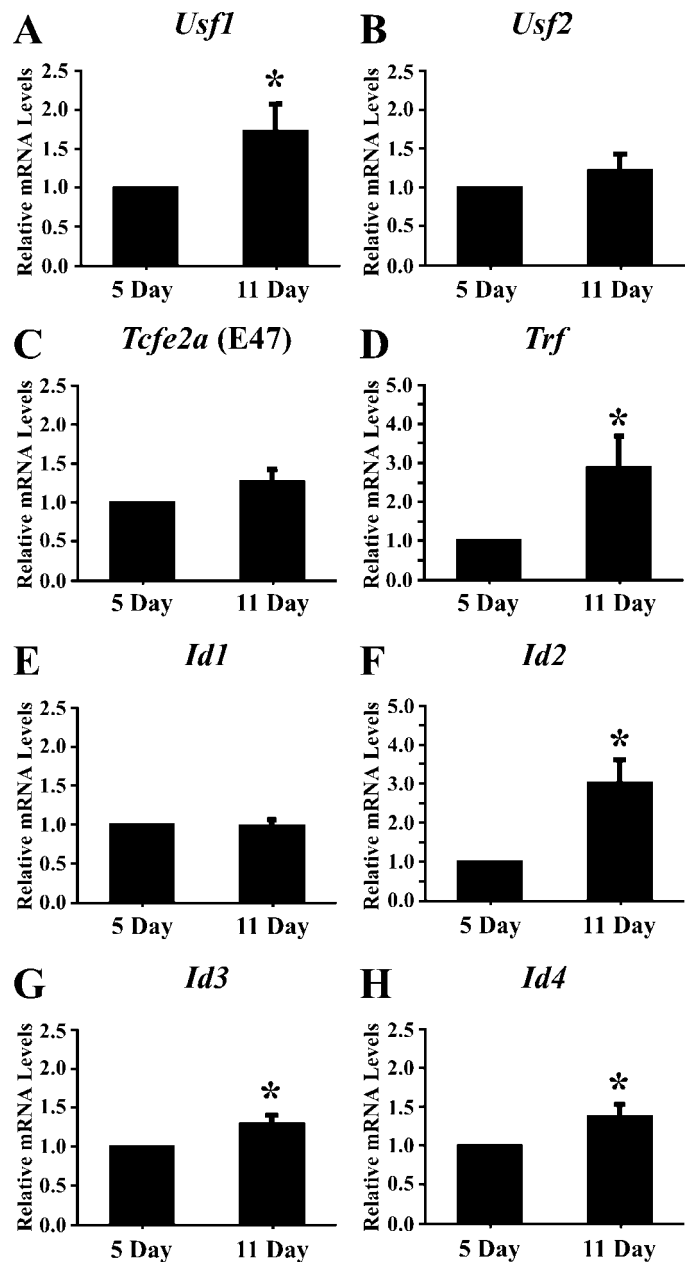


FIG. 5. Expression of E-box and ID protein mRNAs during Sertoli cell development in freshly isolated cells. The mRNAs isolated from 5- and 11-day-old Sertoli cells were analyzed by qRT-PCR using primers for *Usf1* (A), *Usf2* (B), *Tcfe2a* (E47) (C), *Trf* (D), and *Id1*, *Id2*, *Id3*, and *Id4* (E–H). Also analyzed were PCR reactions performed on RT reactions lacking RT (–RT) and PCR reactions performed in the absence of cDNA (no DNA) (data not shown). Data were analyzed using the $\Delta\Delta C_t$ method, and quantitation of the mean (\pm SEM) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and were made relative to 5-day-old Sertoli cells (=1). Statistically significant differences ($P < 0.05$) are indicated by an asterisk (*).

not shown). The use of the efficiency-corrected ΔC_t method allowed us to determine that *Usf2* mRNA levels in Sertoli cells are 4- to 6-fold greater than *Usf1* mRNA levels (Table 3).

Id2, *Id3*, and *Id4* mRNA Levels Increase During Sertoli Cell Differentiation

Increased USF protein binding to E-box motifs during Sertoli cell differentiation could result from decreased

TABLE 3. qPCR primer validation and relative quantity of mRNAs in freshly isolated rat Sertoli cells.

Gene	Efficiency ^a	Mean Ct value in 5-day-old Sertoli cells	Mean Ct value in 11-day-old Sertoli cells	Quantity in 5-day-old Sertoli cells	Quantity in 11-day-old Sertoli cells
<i>Usf1</i>	1.05	25.994 ± 1.5	25.377 ± 1.8	8.62 × 10 ⁻³	1.61 × 10 ⁻²
<i>Usf2</i>	0.90	26.053 ± 1.6	25.929 ± 1.6	5.83 × 10 ⁻²	7.1 × 10 ⁻²
<i>Tcfe2a</i> (E47)	0.99	26.302 ± 1.7	26.111 ± 1.8	1.46 × 10 ⁻²	1.8 × 10 ⁻²
<i>Id1</i>	1.12	28.396 ± 1.4	28.558 ± 1.6	5.75 × 10 ⁻⁴	5.67 × 10 ⁻⁴
<i>Id2</i>	1.01	26.192 ± 1.5	24.770 ± 1.6	1.23 × 10 ⁻²	3.67 × 10 ⁻²
<i>Id3</i>	1.00	26.929 ± 1.3	26.703 ± 1.4	8.61 × 10 ⁻³	1.12 × 10 ⁻²
<i>Id4</i>	1.09	35.679 ± 1.4	35.378 ± 1.2	5.01 × 10 ⁻⁶	6.69 × 10 ⁻⁶
<i>Trf</i>	0.90	27.453 ± 1.8	26.132 ± 1.8	2.36 × 10 ⁻²	6.20 × 10 ⁻²
<i>Ppia</i>	0.93	21.098 ± 1.7	21.241 ± 1.9	1.0	1.0

^a Efficiency determined by a standard curve of input cDNA. See *Materials and Methods* for calculations.

expression of ID proteins that inhibit E-box proteins from dimerizing and binding to DNA [14]. Examination of *Id1* mRNA levels from freshly isolated Sertoli cells revealed no significant differences in the levels of mRNA expression between 5 and 11 days after birth (Fig. 5E), whereas *Id2*, but the levels of *Id1* and *Id4* mRNAs are 100- and 1000-fold lower, respectively (Table 3).

Thyroid Hormone Does Not Greatly Alter the Expression of mRNAs Encoding E-box or ID Proteins

The ability of thyroid hormone to regulate the gene expression of E-box and ID proteins was tested using cultured Sertoli cells. Using qPCR, an initial analysis of basal *Usf1*, *Usf2*, and *Tcfe2a* (E47) mRNA levels in cultured 5- and 11-day-old Sertoli cells indicated that the expression patterns of these mRNAs in cultured Sertoli cells were similar to those found in freshly isolated Sertoli cells (Fig. 6, A–C). In cultured cells, *Usf1*, *Usf2*, and *Tcfe2a* (E47) mRNA levels all increased from 5 to 11 days after birth (41%, 43%, and 59%, respectively) and then decreased to or below the level of 5-day mRNA levels in cells isolated 20 days after birth. *Trf* mRNA levels increased from 5 to 11 days and again from 11 to 20 days after birth, in agreement with the results obtained from freshly isolated cells (Fig. 6D). Thyroxine stimulation (10⁻⁶ M) did not significantly alter the expression of *Usf2*, *Tcfe2a* (E47), or *Trf* but increased *Usf1* mRNA levels by 28% at 11 days. The mRNA levels of the housekeeping gene *Ppia* did not change during the course of differentiation (data not shown).

Basal *Id1* mRNA levels were not altered between 5 and 11 days after birth in cultured Sertoli cells, as was found for Sertoli cells immediately after isolation (Fig. 6E), but between 11 and 20 days after birth, *Id1* mRNA levels decreased significantly (84%). In contrast to the increasing mRNA levels detected in freshly isolated cells, *Id2* mRNA levels remained constant throughout the differentiation period in cultured cells (Fig. 6F). In agreement with the results from the freshly isolated cells, *Id3* mRNA levels increased in cultured Sertoli cells between 5 and 11 days after birth. However, *Id3* mRNA levels decreased between 11 and 20 days after birth, similar to the *Id1* pattern of expression (Fig. 6G). *Id4* mRNA levels in cultured Sertoli cells did not change during the differentiation period, despite showing a small increase between 5 and 11 days in freshly assayed Sertoli cells (Fig. 6H). Stimulation of the cultured Sertoli cells with T4 resulted in a slight further stimulation of *Id2* mRNA levels (24%) at 11 days but not at 5 or 20 days after birth (Fig. 6A). Thyroxine stimulation did not result in any significant changes to *Id1*, *Id3*, or *Id4* mRNA expression at any time point.

Together, the analysis of mRNA levels in freshly isolated and cultured Sertoli cells indicates that most measurements of mRNA expression patterns did not differ greatly for the two model systems. Basal levels of *Usf1*, *Usf2*, *Tcfe2a* (E47), and *Id3* mRNA levels increase between 5 and 11 days after birth, whereas *Id1* and *Id3* mRNA levels decrease between 11 and 20 days during the later stages of Sertoli cell differentiation. *Id2* and *Id4* mRNA levels increase from 5 to 11 days after birth in freshly isolated Sertoli cells, but the levels of these mRNAs do not change significantly in cultured cells. Finally, thyroid hormone has a small positive effect only on *Usf1* and *Id2* mRNA expression and only 11 days after birth.

DISCUSSION

Many differentiation-associated genes are regulated by E-box proteins in Sertoli cells [12, 13, 18, 22, 41, 43, 52, 53]. We have found that during the differentiation of Sertoli cells, E-box protein binding to DNA probes containing a consensus USF-binding site or an *Fshr* promoter E-box increases from 5 to 11 days after birth. The E-box proteins binding to the *Fshr* E-box during differentiation were identified as USF1 and USF2. These findings were similar for Sertoli cells immediately after isolation and for cultured Sertoli cells. These results are consistent with findings by Heckert et al. [13, 41, 53] that USF proteins bound to the *Fshr* promoter E-box in fully differentiated Sertoli cells (27 or 50 days old). We also determined that the DNA-binding activity of the ubiquitous E47 protein was below detection limits in freshly isolated and cultured Sertoli cell nuclear extracts from 5-, 11-, or 20-day-old rats. The inability to detect E47 binding to any of three E47-selective E-box probes is in agreement with findings by Chaudhary and Skinner [22], who noted that E47 binding to the *Trf* promoter E-box was not observed except after stimulation for 48 h by FSH in 20-day-old Sertoli cells. Together, these data indicate that USF1 and USF2, but not E47 or E12 E-box proteins, are potential regulators of Sertoli cell differentiation. The findings that USF proteins are the predominant E-box transcription factors that are able to bind E-box motifs during Sertoli cell differentiation, as well as that USF proteins are the major E-box regulators of *Fshr*, are consistent with previous studies [54, 55] showing that fertility is compromised in the absence of FSHR and USF2. Our data suggest that signals transmitted to the Sertoli cell between 5 and 11 days after birth increase the DNA-binding activity of USF proteins. It is assumed that the increase in USF DNA-binding activity will promote transcription of genes regulated by USF1 and USF2. Therefore, regulatory signals likely act through USF proteins to ensure that the correct differentiation-associated genes are

expressed. USF-induced transcription was not investigated in this study but will be the focus of future work.

It is possible that the increase in USF-binding activity observed during differentiation could be due to an increase in the amount of USF protein present within the cell. Because of the low levels of USF expression in isolated Sertoli cells, Western blot data of whole-cell or nuclear extracts could not be obtained to determine how the levels of these proteins change during Sertoli cell differentiation. However, qPCR was used to evaluate basal mRNA levels for E-box proteins. *Usf1* mRNA levels increased from 5 to 11 days after birth in Sertoli cells assayed immediately after isolation (62%) and in cultured Sertoli cells (41%). *Usf2* and *Tcf2a* (E47) remained unchanged in freshly isolated cells but increased between 5 and 11 days after birth in cultured Sertoli cells (43% and 59%, respectively). It is possible that the increases in *Usf* mRNA levels and any subsequent increased USF protein expression could account for the 2-fold and 7- to 10-fold elevations of USF DNA-binding activity observed during differentiation in freshly isolated and cultured Sertoli cells, respectively. However, it cannot yet be ruled out that another mechanism such as posttranslational modification of USF proteins or activation of accessory proteins is responsible for the increase in USF-binding activity that is observed with the initiation of differentiation.

ID proteins were investigated as potential negative regulators of E-box protein-DNA interactions that would be active before the initiation of Sertoli cell differentiation. However, instead of observing decreased *Id* expression during differentiation, the levels of all *Id* mRNAs increased or remained constant between 5 and 11 days after birth. Because ID proteins block E-box protein function, an increase in expression of the ID proteins would not be expected to contribute to the activation of USF DNA-binding activity found in 11-day-old Sertoli cells. Although ID mRNA expression patterns do not suggest a role for Id proteins in regulating USF-mediated Sertoli cell differentiation 5 to 11 days after birth, the possibility remains that ID proteins regulate other USF-mediated processes in Sertoli cells at other stages of development. Previously, overexpression of ID1 was found to inhibit expression of the USF-regulated *Fshr* promoter [20]. *Id1* and *Id3* mRNA levels decreased dramatically (85% and 70%, respectively) during the later stages of differentiation between 11 and 20 days after birth, but the significance of the decrease in *Id1* and *Id3* expression, as well as any relationship to the activity of the *Fshr* promoter, is not clear at this time. Additional studies are required to determine whether ID proteins directly or indirectly inhibit USF protein and E-box interactions, as well as the regulation of USF-mediated transcription.

Our studies using cultured Sertoli cells indicated that T4 increased the DNA-binding activity of USF proteins in undifferentiated Sertoli cells from 5-day-old rats. The increase in USF protein-DNA interactions in 5-day-old Sertoli cells in response to T4 is further evidence that cells at this early stage of differentiation are receptive to thyroid hormone signals. The timing of the T4-mediated increases in USF DNA-binding activity is consistent with previous data showing that thyroid hormone inhibits Sertoli cell proliferation but promotes differentiation between 4 and 8 days after birth [48, 56, 57]. Thyroid hormone stimulation of cultured Sertoli cells isolated from 11-day-old rats decreased USF binding to a consensus E-box probe and to the *Fshr* promoter E-box probe. This decrease in binding activity occurred in spite of T4-mediated increases in *Usf1* mRNA levels 11 days after birth. It is possible that the small increase in *Usf1* mRNA levels observed after T4

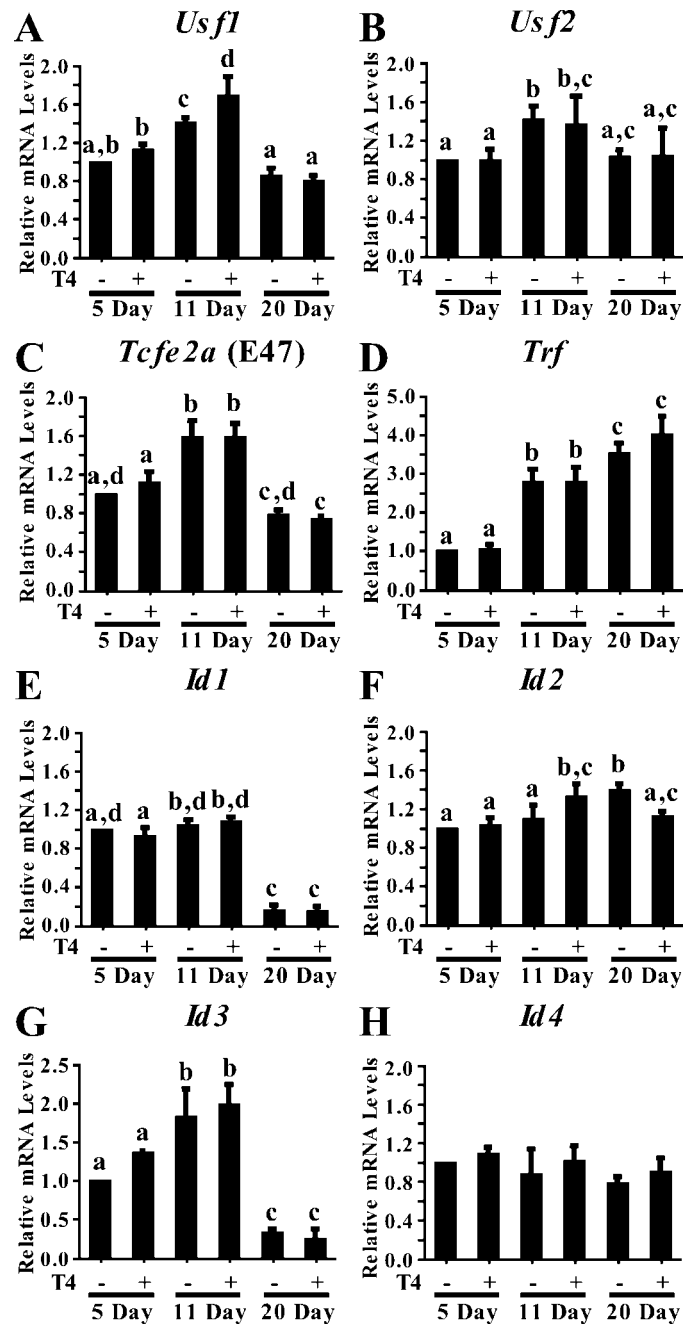


FIG. 6. Expression of E-box and ID protein mRNAs during Sertoli cell development. The mRNAs isolated from cultured 5-, 11-, and 20-day-old Sertoli cells treated with vehicle (–) or T4 (10^{-6} M) for 24 h were analyzed by qRT-PCR using primers for *Usf1* (A), *Usf2* (B), *Tcf2a* (E47) (C), *Trf* (D), and *Id1*, *Id2*, *Id3*, and *Id4* (E–H). Also analyzed were PCR reactions performed on RT reactions lacking RT (–RT) and PCR reactions performed in the absence of cDNA (no DNA) (data not shown). Data were analyzed using the $\Delta\Delta Ct$ method, and quantitation of the mean (\pm SEM) of three individual experiments for each condition is provided for each primer set. The relative mRNA levels were normalized to *Ppia* levels and were made relative to untreated 5-day-old Sertoli cells ($=1$). Values with different lowercase letters differ significantly ($P < 0.05$).

stimulation may be negated by other factors that inhibit USF1 DNA-binding activity by a posttranslational mechanism [58–61]. The exact mechanism of how thyroid hormone contributes to changes in USF activity during the transition from proliferation to differentiation within Sertoli cells remains to be determined.

Identification of the mechanisms that regulate Sertoli cell differentiation is crucial to understanding the establishment of male fertility. To assay the biochemical and gene expression changes that occur during Sertoli cell differentiation, we used two model systems. For the first time (to our knowledge), enriched fractions of proliferating and differentiating Sertoli cells assayed directly after isolation from testis tissue were used to complement studies of cultured Sertoli cells. Comparison of the two models confirmed that Sertoli cells retain their differentiation status in culture because the protein activities and mRNA expression patterns in cultured Sertoli cells closely mimicked those in freshly isolated Sertoli cells. Our results suggest one possible mechanism to initiate the onset of differentiation in Sertoli cells. We propose that signals, including thyroid hormone, initiate a cascade of molecular events that allows transcription factors, including USF1 and USF2 E-box-binding proteins, to activate the transcription of differentiation-promoting genes. The differentiation-promoting genes would then enable the Sertoli cell to support germ cell development and may facilitate exiting the cell cycle. Further characterization of USF1 and USF2 regulation during Sertoli cell development will identify how these bHLH proteins function in the regulation of a molecular switch from proliferation to differentiation in Sertoli cells.

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