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Role of Basic-Helix-Loop-Helix Transcription Factors in Sertoli Cell Differentiation: Identification of an E-Box Response Element in the Transferrin Promoter*

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ABSTRACT

Sertoli cells are critical for testicular function and maintenance of the spermatogenic process. The induction of Sertoli cell differentiation in the embryo promotes testicular development and male sex determination. The progression of Sertoli cell differentiation during puberty promotes the onset of spermatogenesis. The maintenance of optimal Sertoli cell differentiation in the adult is required for spermatogenesis to proceed. The current study was designed to investigate the transcriptional regulation of Sertoli cell differentiation through the analysis of a previously identified marker of differentiation, transferrin gene expression. Sertoli cells produce transferrin to transport iron to developing spermatogenic cells sequestered within the blood-testis barrier.

The transferrin promoter was characterized and found to contain two critical response elements, designated Sertoli element 1 (SE1) and Sertoli element 2 (SE2). Through sequence analysis, SE2 was found to contain an E-box response element, which has been shown to respond to basic-helix-loop-helix (bHLH) transcription factors. The bHLH proteins are a class of transcription factors associated with the induction and progression of cell differentiation. bHLH proteins dimerize through the conserved helix-loop-helix region and bind DNA through the basic region. Nuclear extracts from Sertoli cells were found to cause an E-box gel shift when the cells were stimulated to differentiate in culture, but not under basal conditions. The SE2 gel shift of Sertoli nuclear extracts was competed with excess unlabeled SE2 or E-box DNA fragments. Several Sertoli nuclear proteins associate with the SE2 gel shifts, including 70-, 42-, and 25-kDa proteins. Therefore, the critical SE2 element in the transferrin promoter is an E-box element capable of binding bHLH transcription factors. The ubiquitously expressed E12 bHLH protein dimerizes with numerous cell-specific bHLH factors. A Western blot analysis demonstrated that E12 was present in Sertoli cell nuclear extracts and associated with the SE2 gel shift. A ligand blot of Sertoli cell nuclear extracts with radiolabeled E12 had apparent bHLH proteins when the cells were stimulated to differentiate. The E-box sequence in the SE2 fragment of the transferrin promoter was CATCTG and was similar in gel shifts to the consensus E-box elements (CANNTG) previously characterized. A bHLH inhibitory factor (Id) competed and inhibited formation of the Sertoli cell nuclear extract E-box gel shift. To extend this observation, Id protein was overexpressed in cultured Sertoli cells. A transferrin promoter chloramphenicol acetyltransferase construct was used to monitor Sertoli cell function. The presence of Id suppressed the activation of the promoter induced by Sertoli differentiation factors. Therefore, the inhibition of Sertoli bHLH factors by Id suppressed Sertoli cell differentiated function, as measured by transferrin expression. An E-box-chloramphenicol acetyltransferase construct was also found to be active in Sertoli cells when cells were induced to differentiate. Screening the computerized nucleotide data bases demonstrated that putative E-box response elements are present in the promoters of a large number of Sertoli cell differentiated genes.

In summary, a critical E-box response element has been identified in the transferrin promoter that can be activated by bHLH factors (*e.g.* E12) present in Sertoli cells. Inhibition of Sertoli bHLH factors by Id suppresses Sertoli cell differentiated function (*i.e.* transferrin expression), suggesting that bHLH transcription factors may be important in regulating Sertoli cell differentiated functions. (*Endocrinology* **138:** 667–675, 1997)

THE SERTOLI cells (1, 2) form the basal and apical surface of the seminiferous tubule and provide the cytoarchitectural arrangements for the developing germinal cells (3). Tight junctional complexes between the Sertoli cells contribute to the maintenance of a blood-testis barrier (4) and create a unique environment within the tubule (5). Due to the blood-testis barrier, Sertoli cells produce a number of transport proteins to deliver nutrients to germ cells (6), including transferrin (Tf) to transport iron (7). As the Sertoli cell differentiates during pubertal development, differentiated functions, such as Tf expression, increase and are optimal in the adult to maintain testicular function. Although a number of factors have been shown to influence Sertoli differentiated function, the transcriptional regulation of Sertoli cell differentiation has not been rigorously investigated. Several genes that are apparently involved in initial determination of Sertoli cell fate are SRY (8), Wilms' tumor antigen (9), and possibly Pax-2 (10), but the specific mechanisms and factors involved in detemination of the fate of the Sertoli cell are unknown. FSH has a role in the progression of Sertoli differentiation and can regulate genes through cAMP response elements (11) as well as promote immediate early genes, such as c-*fos* (12–14). Another approach has been to examine the regulation of promoters of Sertoli cell-specific genes, such as Tf (15), FSH receptor (16), and androgen-binding protein (17). However, Sertoli cell-specific transcription factors and response elements remain to be fully elucidated. PModS is a testicular paracrine factor produced by peritubular myoid cells (18, 19) that has been shown to influence Sertoli cell differentiated function (19, 20). Recently, a PModS preparation has been shown to act on the Sertoli cell by inducing c-*fos* expression involving the serum response element of the c-*fos*

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promoter and intermediate transcription factors acting at unique response elements on the Tf promoter (21). The current study characterized these Tf promoter response elements and investigated the intermediate transcription factors. One of the response elements on the Tf promoter was identified as an E-box sequence that binds basic-helix-loop-helix (bHLH) transcription factors.

bHLH proteins are a class of transcription factors previously shown to be involved in cell-specific transcriptional control in a number of tissues, including muscle and brain (22–25). An example in muscle is a family of bHLH transcription factors (*i.e.* the MyoD family) that when expressed are sufficient to orchestrate the coordinated expression of most, if not all, muscle differentiated functions (26). Overexpression of MyoD-related genes can cause a fibroblast cell to turn into a muscle cell (27). Another example is NeuroD, which promotes neural cell differentiation and phenotype (28). These and a number of other observations have suggested that basic HLH transcription factors play important roles during development in many organs. An example relevant to the current study is the requirement for bHLH proteins in sex determination and gonadal development in *Drosophila* (29, 30). This observation provides additional support for the proposed hypothesis of an important role for bHLH factors in Sertoli cell differentiation.

The bHLH proteins have a conserved helix-loop-helix domain essential for dimerization of different bHLH proteins as well as a basic domain that mediates DNA binding to a common hexanucleotide sequence (CANNTG) known as the E-box. An inhibitory class of HLH proteins (Id) lacks the basic region and prevents DNA binding after dimerization with a bHLH protein (31, 32). Therefore, Id negatively regulates bHLH proteins by preventing them from binding to DNA. The bHLH proteins generally form heterodimers between ubiquitously expressed bHLH proteins, such as E12 or E47, and cell-specific bHLH proteins, such as MyoD for muscle development or NeuroD for neural development. The bHLH heterodimer formed will then bind to an E-box response element and promote cell- or tissue-specific gene expression. Often factors that influence cell proliferation will influence bHLH expression (33, 34). The results presented here suggest that hormones and a paracrine factor produced by peritubular cells may influence Sertoli cell differentiated function (*e.g.* Tf expression) through bHLH-type transcription factors.

Materials and Methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (35) with a modified procedure described by Tung *et al.* (36). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Life Technologies, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma Chemical Co., St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were then plated under serum-free conditions in 24-well Falcon plates (Falcon Plastics, Oxnard, CA) at 1×10^6 cells/well. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Life Technologies) with 0.01% BSA at 32 C. Sertoli cell were left untreated (control) or treated with either FSH (100 ng/ml; oFSH-16, National Pituitary Agency) or PModS (S300; 50 μg/ml). These optimal concentrations of FSH and PModS (S300) have previously been shown to dramatically stimulate cultured Sertoli cell differentiated function (19, 20). The cells were cultured under serum-free conditions for a maximum of 5 days, with a medium change and treatment after 48 h of culture. Cell number and viability did not change during culture in the absence or presence of treatment (19, 20).

Plasmids

The E-box-chloramphenicol acetyltransferase (CAT) reporter plasmid was constructed by ligating the Tf E-box oligonucleotide (CATCTG; 15 bp flanking on either side) at the *Hin*dIII site in the pCAT enhancer (Promega, Madison, WI) plasmid. The −581-bp mouse Tf (mTf)-CAT reporter plasmid (pUC8-CAT) and the human GH reporter plasmid containing the −3.0 kilobase (kb) sequence of the mTf promoter were provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA). The CAT reporter plasmid used in the current study containing −2.6-kb mTf promoter was constructed by ligating the 2-kb upstream *Hin*dIII-*Hin*dIII fragment from the 3-kb mTf promoter in the upstream *Hin*dIII site of −581 bp mTf-CAT (21). The pREP Id-1S (Id-1 sense), pREP Id-1AS (Id-antisense), and recombinant E12 were provided by Dr. Jay Cross (McGill University, Montreal, Canada). The GST-Id-2 fusion plasmid was kindly provided by Dr. Mark Israel (University of California-San Francisco).

Transfections and CAT assays

Sertoli cells cultured for 48 h were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described (37) for Sertoli cells (21). Various treatments were subsequently added, and cells were incubated for an additional 72 h before harvesting for CAT assays. Assay of CAT activity was performed with the [^{14}C]chloramphenicol conversion, as previously described (21). The average conversion of CAT substrate for treated cells ranged between 20–30% conversion. This assay was linear with the protein concentration used.

Gel mobility shift assay

Gel shift assays were performed with nuclear extracts of isolated Sertoli cells. The Sertoli cells were isolated as described above and cultured in 150 × 20-mm tissue culture dishes (Nunclon). The cells were treated after 48 h in culture with FSH or S300; controls were not treated. After 72 h, the cells were scrapped off the tissue culture dishes and washed once with PBS. The nuclear extracts of these cells were then prepared as described by Guillou *et al.* (38). Typically, 70–100 μg protein were obtained from 10^8 plated cells. The ^{35}S-labeled nuclear protein was obtained by culturing the cells in Ham's F-12 lacking cysteine and methionine. ^{35}S-Labeled cysteine and methionine were added, and the cells were cultured, and nuclear extracts were prepared as described above. The double stranded DNA probes used in gel retardation assays were the Tf E-box (CATCTG) containing flanking sequence (CCGGGCTCC*ATCTG*CAGCCT), muscle creatine kinase (MCK) E-box (CACCTG) containing flanking sequence (GATCCCCCCAA*CACCT*GCTGCCTGA), mutated Tf E-box (CGCCGG) containing flanking sequence (CGGGCTG*CGCCGG*GAGCCCGG), mutated MCK E-box (CGCCGT) containing flanking sequence (GATCCCCCCAA*CGCCGT*CTGCCTGA), and the Sertoli element 2 (SE2) fragment of the mTf promoter (between −1763 and −1514 bp). The single stranded oligonucleotides were 5′-^{32}P end-labeled with [γ-^{32}P]ATP (150 μCi/μl; New England Nuclear, Boston, MA) and polynucleotide kinase. The complementary oligonucleotides were annealed, electrophoretically purified, and used as probes in gel shift assays. The SE2 fragment was first dephosphorylated with alkaline phosphatase and then phosphorylated using [γ-^{32}P]ATP (150 μCi/μl) and polynucleotide kinase. The labeled fragment was electrophoretically purified and used as a probe.

The gel retardation assay used was a modification of the protocol described by Garner and Rezvin (39). The final reaction volume of 20 μl contained 0.5 ng (~50,000 cpm) 5′-^{32}P-labeled double stranded probe, 100 ng sonicated salmon sperm DNA, 2 μg poly(dI-dC) (U.S. Biochemical Corp., Cleveland, OH), 20 μg BSA, 20 mM HEPES (pH 8.0), 4 mM Tris (pH 7.9), 50 mM KCl, 600 μM EDTA and EGTA, 500 μM dithiothreitol, and 5 μg Sertoli cell nuclear proteins. After incubation at room temperature for 20 min, 5 μl of the reaction were electrophoretically separated on a nondenaturing 5% polyacrylamide gel in 0.5 × TBE. The

gel was dried and autoradiographed. For the competition experiments, excess unlabeled oligonucleotide was added in the binding reaction. To analyze the number of proteins binding to the Tf E-box, gel shift was performed using ^{35}S-labeled nuclear extracts from Sertoli cells treated with S300. The retarded gel shift band was isolated, and the protein-DNA complexes within gel slices were then resolved by SDS-PAGE and visualized by autoradiography.

Ligand and Western blots

Ten micrograms of Sertoli cell nuclear proteins were size-fractionated on a 12% discontinuous SDS-polyacrylamide gel. The proteins were then transferred to 0.2-μm nitrocellulose membranes (BA83, Schleicher and Schuell, Keene, NH). These membranes were used for ligand and Western blots.

Ligand blot. A ligand blot using labeled E12 was used to study the presence of bHLH proteins in Sertoli cell nuclear extracts. The membrane was blocked with 5% milk-HBB buffer (20 mM HEPES, pH 7.7; 5 mM $MgCl_2$; 25 mM NaCl; and 1 mM dithiothreitol) before probing with recombinant γ-^{32}P-labeled E12 in Hyb75 buffer (20 mM HEPES-KOH, pH 7.7; 75 mM KCl; 0.1 mM EDTA; 2.5 mM $MgCl_2$; 1% milk; 1 mM dithiothreitol; and 0.05% Nonidet P-40). The recombinant E12 fusion protein contained the bHLH domain of hamster sh PAN-2 (amino acids 509–649 with mutations R554A and R556A) fused to a heart muscle kinase recognition sequence (40). This E12 fusion protein was phosphorylated by heart muscle kinase in the presence of [γ-^{32}P]ATP.

Western blot. Western blot analysis using an antibody to human E12 (SC 762, Santa Cruz Biotechnology, Santa Cruz, CA) was performed to identify E12 in Sertoli cell nuclear extracts. The membranes were probed for 3 h at room temperature with the E12 antibody. Antigen-antibody complexes were identified using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

To determine whether E12 is one of the components of the complex binding to the E box during gel shifts, the retarded gel shift band was excised and loaded onto a 12% SDS-polyacrylamide gel. The size-fractionated proteins were then transferred to a 0.2-μm nitrocellulose membrane and probed with E12 antibody as indicated above.

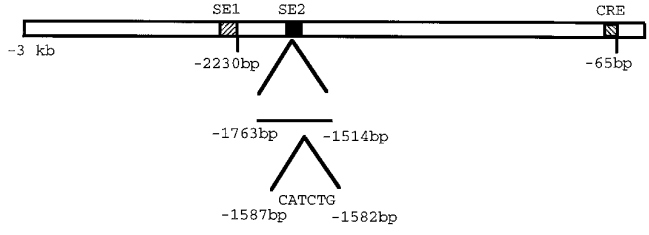
Statistical analysis

All data were obtained from a minimum of three different experiments unless otherwise stated. Each data point was converted to a relative CAT activity, with the mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by Student's *t* test as indicated in the figure legends.

Results

Previous analysis of the 3-kb mTf promoter and its deletion mutants suggested that an upstream 1-kb fragment between −2.5 to −1.5 kb may have an important role in the Sertoli cell-specific expression of the Tf gene (21). Within this 1-kb region, two potential *cis*-acting elements were identified, termed SE1 and SE2, that complexed Sertoli cell nuclear proteins when the cells were stimulated with differentiation factors. To further analyze these response elements, the mTf promoter was sequenced. Interestingly, a nonpalindrome E-box sequence (CATCTG), termed Tf E-box, was identified in SE2 located between −1587 and −1582 bp (Fig. 1). SE2 did not contain any other common response element sequences. A comparison of the Tf E-box sequence to other known E-box sequences (consensus CANNTG) revealed its presence in other promoters, such as the c-*fos* promoter, which can respond to bHLH (41, 42). A computerized data bank search revealed that putative E-box response element sequences are present in the promoters of a number of Sertoli cell-specific genes (Table 1). However, the functional significance of these E-box elements remains to be determined. These genes may have the ability to respond to bHLH proteins, but this needs to be investigated.

To address the question of whether bHLH proteins are present in Sertoli cells, gel shift experiments were initially performed. As shown in Fig. 2, a gel shift was observed when a radiolabeled Tf E-box oligonucleotide, including flanking region, was used as a probe in the binding reaction with nuclear extracts from PModS (S300)- and FSH-stimulated Sertoli cells. PModS (S300) is a gel filtration fraction preparation of PModS from serum-free concentrated peritubular cell secreted proteins that has been shown previously to dramatically stimulate Sertoli cell differentiated functions



SE2 Nucleotide Sequence

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-1763bp AGATGTGAAGATGAGAGGGTGGCGCCACAACGTTCAGTGTTCTTGGTGG
        TCCAAGACACATCTGCCATGTCCCAATTTTGTGTGTTTAATTCCACACC
        AGAGAGCTTCTGGTCACCCACGGCTCCTCTTGAACACCTTAGCTATGGC
        TGAGACGTGCTGAGAGCTTCACCGGGCTCCATCTGCAGCCTGAAATCAG
        AGCGTCAACATCCTGCAGACTTGACAAGGTAAACCGAGGACCAGAGAGG
        ACAGG -1514bp
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FIG. 1. Sequence of SE2 of the mTf promoter. SE2 is located between −1763 and −1514 bp upstream of the transcriptional start site on the promoter. The relation to SE1 and the cAMP response element (CRE) is shown. The E-box response element (CATCTG) located in SE2 is *underlined*.

TABLE 1. Putative E-box response elements in promoter regions of genes involved in Sertoli cell differentiation

Promoter	Species	E-box sequence	Location (bp)
Transferrin	Murine	CATCTG	−1582
FSH receptor	Murine	CACGTG	−261
Androgen binding protein	RAT	CATCTG	−1317, −129
Inhibin (β-subunit)	RAT	CATTTG	−106
Inhibin (α-subunit)	RAT	CACGTG	−84
Mullerian inhibiting substance	Bovine	CAGCTG	−479
	RAT	CAGGTG	−1322, −809, −206
		CACCTG	−1089, −650, −101
		CATCTG	−961, −898
SRY (sex-determining gene)	Murine	CACATG	−65
	bovine	CATCTG	−537
		CAGCTG	−188

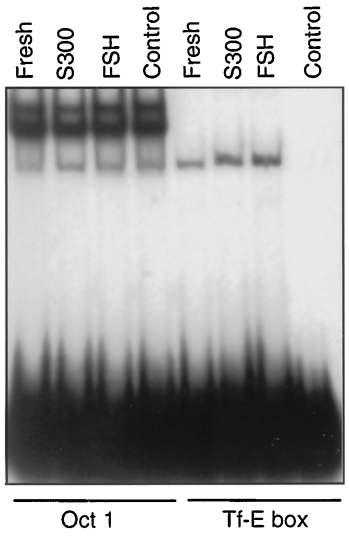

FIG. 2. A Tf E-box (Tf-E-box) gel shift with Sertoli cell nuclear extracts from control unstimulated cells, PModS (S300)- or FSH-treated cells, and freshly isolated Sertoli cells from 20-day-old rats (Fresh). *Right*, The gel shift with the Tf-E-box; *left*, the gel shift with Oct-1 oligonucleotide to confirm nuclear extract integrity. The gel shift is representative of a minimum of three different experiments.

(21). The presence of a single shifted band suggests that FSH and PModS (S300) promote and maintain the expression of Sertoli cell E-box binding proteins, which will include bHLH proteins. A similar band shift was not observed in untreated control Sertoli cells cultured in the absence of FSH or PModS (S300). However, an Oct-1 gel shift was observed, demonstrating the integrity of the nuclear extracts. The transcription factors that bind an Oct-1 oligonucleotide are abundant and provide a useful procedure to monitor the integrity of nuclear extracts and help normalize gel shift data. As previously shown, Sertoli cell number and viability did not change during the culture period whether cells were cultured in the absence or presence of FSH or PModS (S300). An E-box gel shift was also detected in the nuclear extracts of Sertoli cells freshly isolated from 20-day-old midpubertal rats (Fig. 2). Therefore, the lack of a gel shift in control cultured Sertoli cells was due to an apparent loss of bHLH proteins during culture. Sertoli cell differentiated functions are known to decline in control untreated cultures (19, 20). The specificity of the gel shift was determined by using excess unlabeled Tf E-box oligonucleotide in the binding reaction. As shown in Fig. 3, the unlabeled Tf E-box oligo at a 100-fold molar excess was able to completely abolish the observed gel shift. Concentrations of unlabeled competitor less than a 100-fold molar excess resulted in partial displacement (data not shown). Excess unlabeled SE2 was also able to displace the gel shift observed with labeled Tf E-box (Fig. 3A). As previously shown (21), the SE2 fragment of the Tf promoter located at −1763 to −1514 bp was able to cause a gel shift when incubated with nuclear extracts of Sertoli cells treated with PModS (S300). This shift appeared to be due to the Tf E-box sequence (CATCTG) present in SE2 between −1587 bp to −1582 bp. Addition of excess unlabeled Tf E-box in the binding reaction abolished the SE2 gel shift (Fig. 3B). Excess unlabeled Oct-1 had no effect on the gel shift (data not

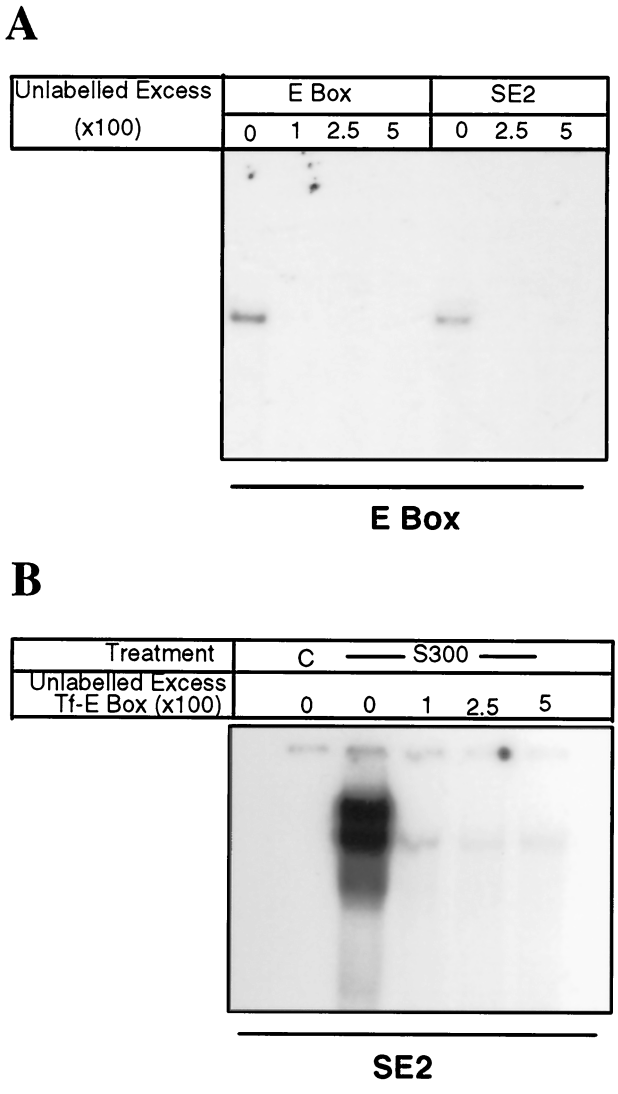

FIG. 3. A, A Tf E-box (E-box) gel shift with Sertoli cell nuclear extracts from PModS (S300)-treated cells. The gel shift was displaced with excess unlabeled E-box oligonucleotide duplex DNA or SE2 (200-bp) DNA fragment. The molar ratio of unlabeled excess DNA fragment is indicated. The gel is a representation of a minimum of three different experiments. B, An SE2 gel shift with Sertoli cell nuclear extracts from PModS (S300)-treated cells. The gel shift was displaced with excess unlabeled Tf E-box (Tf-E-box) with the molar excess indicated. The gels are representative of a minimum of three different experiments.

shown). Taken together, the observations suggest that the gel shift observed with SE2 is due to the Tf E-box sequence. Whether the multiple bands in Fig. 3B were due to proteolytic degradation of the nuclear extract or different protein shifts remains to be determined; however, all were competed with the unlabeled E-box.

To characterize the E-box binding proteins in the gel shift, the proteins associated with the Sertoli cell E-box gel shift were examined. The gel shift was performed with [^{35}S]cysteine- and [^{35}S]methionine-radiolabeled nuclear extracts from Sertoli cells. The shifted band from the gel shift experiment was isolated and loaded onto a 12% polyacrylimide-SDS gel. A fluorograph of the gel revealed three consistently

detected proteins of 70, 42, and 25 kDa (Fig. 4). A control of isolating a comparable band from a displaced gel shift was also run and showed no radiolabeled proteins (data not shown). The width of the well and the salt concentration needed to permit loading of sufficient amounts of sample caused a distortion in the bands compared with crude extracts. The E-box proteins detected were not prominent bands in the crude extract. The observation suggests that multiple proteins appear to associate and bind to the Tf E-box oligo nucleotide duplex. However, the specificity of binding of these various proteins needs to be determined. A Western blot of this gel with an E12 antibody revealed that the 70-kDa protein was E12 (Fig. 4). A smaller 29-kDa band observed in the Western blot of the crude extract was probably a proteolytic fragment of E-12 not capable of binding the E-box. Recently, we also showed the presence of E12 by reverse transcription-PCR in Sertoli cells using rat E12 primers (data not shown). The 42- and 25-kDa proteins remain to be identified. The bands observed at 14 kDa may be a proteolytic fragment of the larger proteins and was not always detected. Observations from the SDS gel imply that multiple proteins may be involved in the Tf E-box gel shift.

An alternate procedure to identify bHLH proteins in Sertoli cells involved a ligand blot with a radiolabeled bHLH protein. The Sertoli cell nuclear extracts were electrophoretically separated on an SDS gel, blotted onto nitrocellulose, and probed with recombinant radiolabeled E12. E12 is a ubiquitously expressed bHLH protein found in many tissues and forms heterodimers with cell-specific bHLH proteins. As shown in Fig. 5, a number of different bands were observed, indicating that Sertoli cells contain proteins that can dimerize with E12. A control with denatured E12 showed no nonspecific binding (data not shown). The demonstration of specific binding with displacement of unlabeled E12 remains to be performed. This observation supports the E-box gel shift data and also suggests that bHLH proteins may be present in Sertoli cells.

The Tf E-box has the E-box consensus sequence, but is not palindromic like the more classical CACCTG or CAGCTG found in the enhancer of MCK (43) and troponin-1 (44). To investigate whether the bHLH binding properties to the classical (CACCTG) E-box of MCK are similar to those of the Tf (CATCTG) E-box, gel shifts were performed in which the MCK E-box, including flanking region, was used as a probe, and excess unlabeled Tf E-box was added in the binding reaction. As shown in Fig. 6, a gel shift was observed when either MCK E-box or Tf E-box was used as probes, and the gel shifts could be displaced by adding excess unlabeled MCK E-box or Tf E-box. This suggests that the Sertoli bHLH

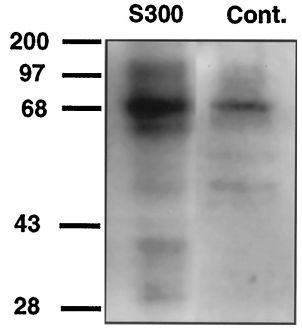

FIG. 5. An E12 radiolabeled ligand blot of Sertoli cell nuclear extract proteins electrophoretically separated on a SDS gel and autoradiographed. The Sertoli cell nuclear extracts were isolated from control untreated (Cont) and PModS (S300)-treated cells. The molecular size (kilodaltons) is listed at the *left*. The gel is representative of three different experiments.

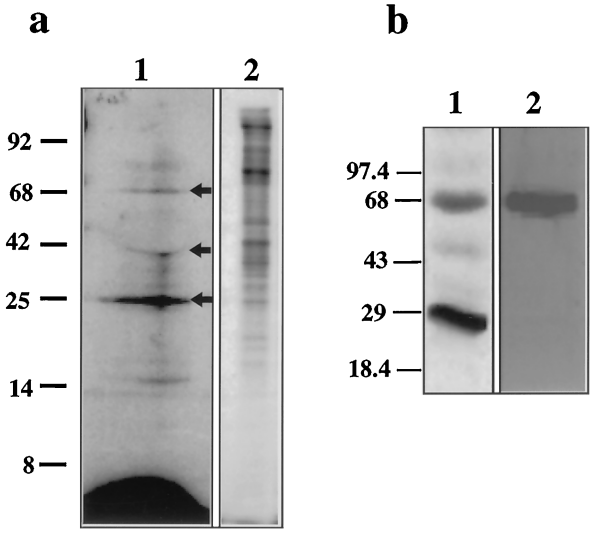

FIG. 4. A, The radiolabeled (^{35}S-Cys/met) Sertoli cell nuclear extract proteins that associate with a Tf-E-box gel shift electrophoretically separated on a SDS gel and fluorographed. The gel shift-associated proteins are shown in lane 1 (*wide lane*), and the total nuclear extract is shown in lane 2. B, Western blot analysis of Sertoli cell nuclear extracts (lane 1) and the retarded band of the gel shift assay (lane 2; see Fig. 2) using antibody to human E12. The molecular size in kilodaltons is shown at the *left*. The gel is representative of three different experiments.

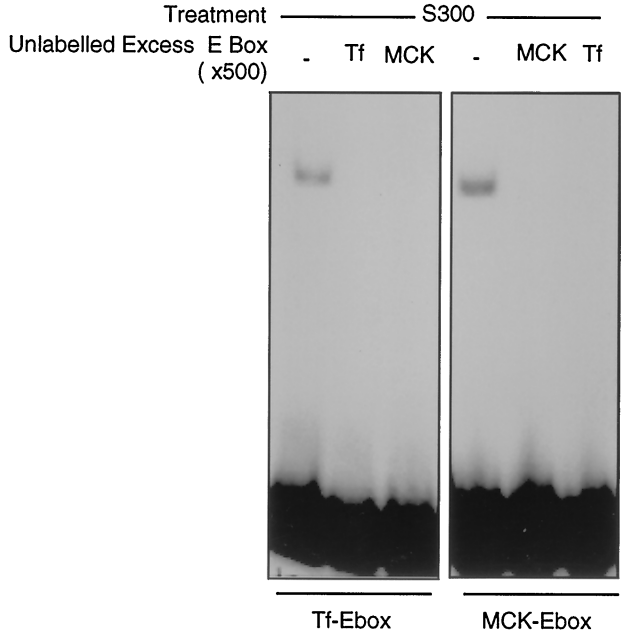

FIG. 6. A gel shift with Tf-E-box or MCK-E-box with Sertoli cell nuclear extracts from cells treated with PModS (S300). Excess unlabeled Tf-E-box (Tf) or MCK-E-box (MCK) is indicated and displaced the gel shift. The gel is representative of three different experiments.

proteins have binding properties to the Tf E-box similar to those to the MCK E-box and further strengthens the observations that the Tf E-box is active, and bHLH proteins are present in Sertoli cells.

Further analysis of the specificity of the Sertoli cell E-box gel shift used a mutated E-box (CGCCGG) that had alterations in the critical (CANNTG) E-box sequence. This was performed with both the Tf E-box and MCK E-box sequences. As shown in Fig. 7, these mutated E-box oligonucleotide duplex DNA fragments could not promote a gel shift with Sertoli cell nuclear extracts. The mutated E-box fragments also could not displace the Sertoli bHLH gel shifts promoted with intact Tf E-box or MCK E-box sequences (Fig. 7). Therefore, the critical nucleotides in the (CANNTG) E-box consensus sequence are functionally important for the Tf E-box sequence.

To investigate the functional significance of bHLH proteins in Sertoli cells, the inhibitory bHLH protein Id was used. Recombinant Id protein was produced in a glutathione transferase (GST) fusion protein bacterial system. The recombinant GST-Id protein was added to the binding reaction of a Sertoli E-box gel shift experiment. As shown in Fig. 8, the presence of the GST-Id inhibited the ability of a Tf E-box gel shift to form. The GST-Id protein alone also did not cause a gel shift, as expected. A control gel shift with Oct-1 was not effected by the GST-Id construct. Therefore, the presence of Id can prevent the Sertoli bHLH proteins from binding to the Tf E-box. This information was then used to design an experiment to inhibit Sertoli bHLH protein actions *in vitro*.

To assess the functional significance of bHLH proteins in Sertoli cell differentiation, the Sertoli cells were cultured and transfected with a Tf promoter CAT construct (Tf-CAT) as a marker of Sertoli cell differentiation. The cells were also cotransfected with an Id expression plasmid driven by the retroviral RSV promoter to overexpress Id in the Sertoli cells. As a control, both sense Id that generates a functional protein and antisense Id that generates a nonfunctional protein were used. As expected, PModS (S300) stimulated the Tf-CAT activity, reflecting its ability to promote Sertoli cell differentiation (Fig. 9). Relative CAT activity is based on levels of CAT in transfected control unstimulated cells set at 1.0. When Id was overexpressed in the Sertoli cells, approximately a 50% inhibition in the ability of PModS (S300) to activate Tf-CAT was observed (Fig. 9). In contrast, the control

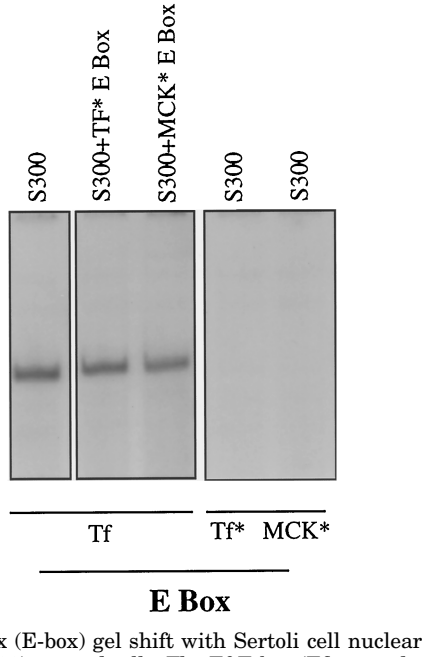

FIG. 7. An E-box (E-box) gel shift with Sertoli cell nuclear extracts from PModS (S300)-treated cells. The Tf-E-box (Tf) caused a gel shift that could not be displaced with mutated Tf-E-box (Tf*E-box) or mutated MCK-E-box (MCK*E-box). The mutant Tf E-box (Tf*) or MCK-E-box (MCK*) also did not cause a gel shift. The gel is representative of two different experiments.

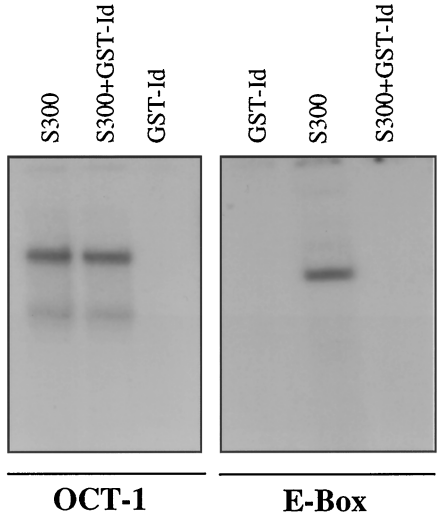

FIG. 8. A Tf E-box gel shift with Sertoli cell nuclear extracts from cells treated with PModS (S300) and incubated in the absence (S300) or presence of GST-Id (S300+GST-Id). GST-Id alone did not cause a gel shift. The data are representative of two different experiments.

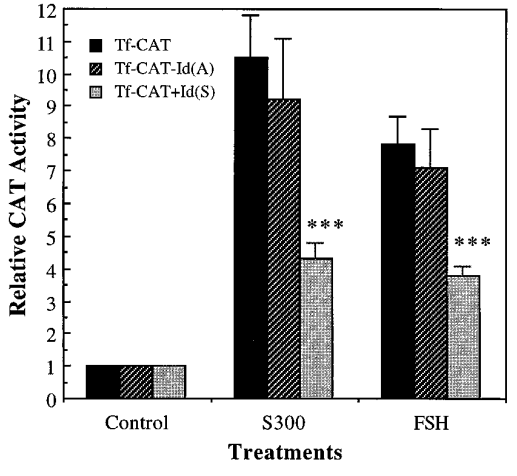

FIG. 9. The expression of a Tf promoter-CAT (Tf-CAT) construct in Sertoli cells cultured in the absence (Control) or presence of PModS (S300) or FSH. CAT activity is expressed as the relative CAT activity based on the CAT activity in control untreated cultures, which was set at 1.0. The cells were transfected with Tf-CAT alone or with Tf-CAT plus Id-sense (Id-S) or Id-antisense (Id-AS) expression plasmids. This is the mean ± SEM from three different experiments, performed in duplicate. **, $P < 0.01$; ***, $P < 0.001$ (statistically significant difference from Tf-CAT alone within the treatment group).

antisense Id had no effect on PModS (S300)-induced Tf-CAT activity. Similar observations were found with FSH-stimulated Sertoli cells (Fig. 9). Therefore, the presence of Id inhibited the Sertoli cell E-box binding proteins (*e.g.* bHLH) from binding to the Tf E-box and activating the Tf promoter.

Another experiment to assess the functional significance of bHLH proteins in Sertoli cells involved the transient transfection of an E-box CAT construct. CAT activity is under the control of the E-box and the binding of bHLH proteins. Cultured Sertoli cells were treated with regulatory agents to monitor the E-box CAT activity. As found with the Tf promoter CAT construct above, both FSH and PModS (S300) stimulated E-box CAT activity (Fig. 10). A CAT plasmid not containing the E-box was also transfected as a control (data not shown), and the CAT activity of this control plasmid was subtracted from the CAT activity associated with the E-box CAT construct. The stimulation of the E-box-CAT construct (Fig. 10) confirms that FSH and PModS (S300) can induce the apparent production and/or activation of Sertoli cell bHLH proteins and that the Sertoli cell bHLH proteins identified are functionally active. This observation also supports a role for bHLH proteins in the control of Sertoli cell differentiated functions.

Discussion

During embryonic development the Sertoli cells are derived from a mesenchymal to epithelial transition in the bipotential gonad. This initial induction of Sertoli cell differentiation involving SRY is one of the first steps in male sex determination and testicular development. Subsequently, the Sertoli cells form sex cords and produce essential components, such as Mullerian inhibitory substance, to promote male reproductive tract development. After embryonic development, the Sertoli cells prepubertally are in a growth phase and maintain a low level of differentiated function. The progression of Sertoli cell differentiation is promoted at the onset of puberty. Differentiated functions increase to an optimal level that is maintained in the adult to control testicular function and the process of spermatogenesis. Whether the induction of Sertoli cell differentiation during embryonic development is distinct or involves similar mechanisms as the progression and maintenance of differentiation during pubertal development and in the adult remains to be elucidated. The current study used midpubertal rat Sertoli cells and, thus, directly relates to the progression and maintenance of Sertoli cell differentiation. Future studies will be required to make comparisons with the induction of differentiation in the embryo. A preliminary experiment, however, demonstrated that bHLH proteins were present in the embryonic testis using the SE2 gel shift (45).

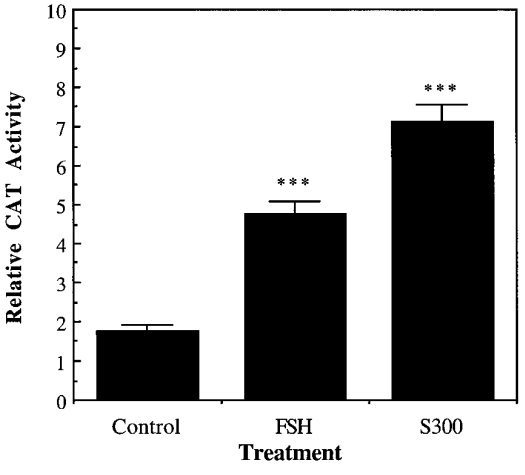

FIG. 10. The expression of an E-box-CAT construct (p-CAT Enh E-box) in Sertoli cells cultured in the absence (Control) or presence of FSH (FSH) or PModS (S300). CAT activity is expressed as the relative CAT activity based on that in control untreated cultures, which was set at 1.0. A representative of three experiments is presented. ***, $P < 0.001$ (statistically significant difference from E-box CAT activity in control cultures).

Sertoli cells have a large number of differentiated functions that generally increase to optimal levels during pubertal development. Previously, Tf expression was shown to be a useful marker of Sertoli cell differentiation and is stimulated by regulatory agents known to promote Sertoli cell differentiation (7). To investigate the transcriptional regulation of Sertoli cell differentiation, the promoters for various Sertoli cell genes have been analyzed, including the Tf promoter (15, 46–49). Analysis of the proximal 600-bp human Tf promoter demonstrated a distinct regulation between hepatocytes and Sertoli cells (48, 49). The current study investigated the 3-kb mTf promoter and focused on two response elements previously shown to be critical, termed SE1 and SE2 (21, 45). Previously, the peritubular cell product PModS was found to promote a gel shift and activate SE1 and SE2 (21). In the current study, FSH was also found to promote a gel shift with SE2. Sequence analysis demonstrated that SE1 did not contain any known response element sequences (data not shown); however, SE2 was found to contain an E-box (CATCTG) sequence.

The E-box is the response element used by the bHLH class of transcription factors. The Sertoli cell nuclear extract SE2 gel shift could be displaced with a consensus E-box oligonucleotide. In addition, a consensus E-box oligonucleotide promoted a gel shift that could be displaced with SE2. A mutant E-box could not promote a gel shift or displace the SE2 or E-box gel shifts. Therefore, the E-box appears to be the response element in SE2 responsible for the gel shift and activation of the Tf promoter. Previously, deletion mutant analysis demonstrated that a promoter construct containing SE2 was the most active region in the Tf promoter (21). Whether SE2 may interact with other elements, such as SE1, remains to be investigated. Interestingly, a computerized data search of the promoter sequences of a number of other Sertoli differentiated genes demonstrated that putative E-box elements are present in all of those examined. This included embryonic genes, such as SRY and Mullerian inhibitory substance, and adult functional genes, such as androgen-binding protein and inhibin/activin. The demonstration of a critical E-box element in the Tf promoter as well as potential elements in other Sertoli cell genes led to the hypothesis that bHLH proteins may be involved in Sertoli cell differentiation.

The bHLH class of transcription factors has been shown to induce and regulate the differentiation of a number of tissues and cell types (22–25). Examples include the MyoD family,

which promotes muscle cell differentiation (22, 23, 26), and NeuroD, which promotes neural cell differentiation (28). Therefore, cell-specific bHLH proteins can promote cell-specific differentiation. The possibility that Sertoli cell differentiation may be influenced by bHLH proteins was initially investigated by identifying the presence of Sertoli cell bHLH proteins. The ability of Sertoli cell nuclear extracts to promote an E-box gel shift confirmed the presence of bHLH proteins in Sertoli cells. Both PModS (S300)- and FSH-treated Sertoli cells promoted an E-box gel shift, whereas control nontreated cultures had no gel shift. This observation supports a role for bHLH proteins in Sertoli cells, as both PModS (S300) and FSH influence Sertoli cell differentiation. The midpubertal 20-day-old rat Sertoli cells are differentiated *in vivo*, so they would be expected potentially to have bHLH proteins. Nuclear extracts from freshly isolated Sertoli cells did cause an E-box gel shift. Therefore, nontreated control cultured Sertoli cells appear to have lost the ability to produce the bHLH proteins that promote the gel shift. This correlates with the low level of differentiated function in control Sertoli cell cultures previously identified (19–21).

The E-box element in the Tf promoter was found to be nonpalindromic (CATCTG) compared with the E-box in MCK (CACCTG), which is palindromic. Previously, bHLH proteins have been classified according to the ability to bind specific E-box sequences (50, 51). Class A binds predominantly palindromic sequences (*e.g.* MyoD family), and class B binds predominantly nonpalindromic sequences (*e.g. myc*). Class C has unique structural properties in the bHLH domains involving the presence of proline residues (*e.g.* HES1 and Hairy) (51). The ability of the Sertoli cell bHLH proteins to bind the nonpalindromic Tf E-box in a similar manner as the palindromic MCK E-box suggests that the Sertoli bHLH proteins have both class A- and B-type bHLH binding properties. Further characterization of the Sertoli cell bHLH proteins is necessary to confirm the class to which these bHLH proteins belong.

The ligand blot suggested the presence of bHLH proteins in Sertoli cells and that FSH and PModS (S300) can stimulate the presence of E-12 binding proteins in Sertoli cells. The E-box gel shift contained at least three radiolabeled Sertoli cell nuclear proteins of 70, 42, and 25 kDa. Whether all of these proteins are bHLH proteins, whether some are not bHLH proteins but associate with the protein complex, and/or whether the small proteins are proteolytic products of the larger proteins remains to be investigated. Previously characterized bHLH proteins range from 15–50 kDa in size. The 70-kDa protein was probably E-12, as demonstrated in the Western blot. These observations imply that bHLH proteins are present in Sertoli cells, and they have the ability to heterodimerize with E12 and bind to an E-box.

The functional significance of Sertoli bHLH proteins was investigated with the use of the inhibitory bHLH protein Id. Id lacks a basic region, so upon dimerization with bHLH proteins, it prevents binding to DNA. Initial experiments with recombinant Id demonstrated that the presence of Id displaced the Sertoli cell bHLH proteins from the Tf E-box gel shift. Therefore, Id can act as an inhibitory bHLH protein in Sertoli cells. An expression plasmid was then transfected into Sertoli cells to overexpress Id. Overexpression of Id was found to inhibit the ability of both FSH and PModS (S300) to stimulate Sertoli cell differentiation, as measured with a Tf promoter-CAT construct. This provides direct evidence that the Sertoli bHLH proteins are involved in the promotion of Sertoli cell differentiation. An additional experiment used an E-box-CAT construct transfected into Sertoli cells. Both FSH and PModS (S300) stimulated the E-box CAT activity. Therefore, the E-box in Sertoli cells is active and induced when the cells are stimulated to differentiate.

Combined observations demonstrate that the bHLH proteins present in Sertoli cells have a functional role in regulating Sertoli cell differentiation, as measured by Tf gene expression. The current study provides information regarding the progression of Sertoli cell differentiation during pubertal development and the maintenance of differentiation in the adult. Whether bHLH proteins have an important role in embryonic Sertoli cell differentiation is currently under investigation. Characterization of the bHLH proteins in Sertoli cells is also currently under investigation. It will be of particular interest to detemine whether Sertoli cell-specific bHLH proteins are present and promote Sertoli cell differentiation. Further analysis of the Sertoli cell bHLH proteins and their roles in the induction, progression, and maintenance of differentiation is anticipated to provide insight into the transcriptional control of Sertoli cells.

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