

E-Box and Cyclic Adenosine Monophosphate Response Elements Are Both Required for Follicle-Stimulating Hormone-Induced Transferrin Promoter Activation in Sertoli Cells*

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ABSTRACT

Sertoli cells are the epithelial cells responsible for the onset of pubertal development and maintenance of spermatogenesis in the adult. Transferrin is one of the major secretory products expressed by differentiated Sertoli cells. Investigation of the transcriptional control of transferrin gene expression provides insight into the regulation of Sertoli cell differentiation. Analysis of the mouse transferrin (mTf) promoter reveals the presence of a number of conserved response elements that have previously been shown to regulate cell specific expression of the human transferrin (hTf) promoter. One of these elements is the human P_{RII} region, which is a cAMP response element (CRE)-like element that is more than 80% conserved in the mTf promoter. The activation of the hTf promoter by FSH and cAMP in rat Sertoli cells has been shown to be mediated in part through the CRE-like P_{RII} region and binding of the CRE binding protein (CREB). The present study investigates the role of P_{RII} in the activation of mTf promoter by FSH and cAMP in rat Sertoli cells. Mutations in the P_{RII} of the mTf promoter reduced FSH activation by only 50% and cAMP activation by more than 90%. In contrast, the mutant P_{RII} mTf promoter construct was fully activated by a partially purified testicular paracrine activity PModS(S300). Gel shift experiments demonstrated that proteins that can bind a consensus CRE oligonucleotide

also bind the P_{RII} region of the mTf promoter. An immunoblot confirmed that CREB binds the P_{RII} and promotes the gel shift observed. The hypothesis developed was that another cis-acting element in addition to the CRE-like P_{RII} is also involved in FSH actions. A conserved response element in both the mTf and hTf promoters is the basic helix-loop-helix (bHLH) responsive E-box sequence. Both FSH and PModS (S300) activity were found to promote a mTf E-box gel shift that contained the E2A gene product the bHLH protein E47. Interestingly, mutations in the E-box of the mTf promoter completely abolished the PModS(S300) activation and partially (52%) inhibited the activation by FSH. In contrast, the mutant E-box mTf promoter construct was fully activated by cAMP. Finally a double mutation of both the P_{RII} and the E-box completely abolished FSH activation of the mTf promoter. These results suggest that optimal activation of the mouse transferrin promoter by FSH requires both CREB binding to the CRE-like P_{RII} region and bHLH binding to the E-box. Information is provided that indicates a number of Sertoli cell promoters contain a close association of E-box and CRE-like elements. Observations are discussed in regards to the potential interactions of the CRE and E-box response elements in mediating FSH actions in Sertoli cells. (*Endocrinology* 140: 1262–1271, 1999)

MULTIPLE INTERACTIONS ARE known to occur between several classes of transcription factors to ensure efficient and cell specific transcription. The cell specific expression of genes is likely mediated in part through interactions between critical *trans*-acting factors. The molecular mechanisms underlying Sertoli cell specific gene expression are poorly understood. The transferrin gene provides an excellent model to elucidate the regulatory mechanisms controlling the expression of a gene in different cell types (1). In contrast to the liver (2), the transferrin gene expression in Sertoli cells is not regulated by iron, but instead by hormones such as FSH (3, 4). Transferrin messenger RNA (mRNA) is present in Sertoli cells of postnatal 5-day-old rats, but the protein is primarily detectable at the onset of puberty (5). This correlates with the differentiation of Sertoli cells and provides a useful marker of Sertoli cell differentiation (6). Investigation of the transcriptional control of the transferrin

gene provides insight into the hormonal regulation of Sertoli cell differentiation.

The primary hormone found to influence Sertoli cell differentiation is FSH. Locally produced paracrine factors also influence Sertoli cell differentiation. The bioactive peritubular cell secretory product termed PModS acts on Sertoli cells in a manner distinct from FSH and does not involve the cAMP-protein kinase A (PKA) pathway (7). The PModS activity in the peritubular cell secretory products, PModS(S300), is a useful agent to stimulate transferrin promoter activation independent of cAMP and FSH. Therefore, the current study compares FSH and PModS(S300) activation of the transferrin promoter to provide insight into FSH actions.

Expression data on the human transferrin promoter in rat Sertoli cells suggested that a minimal promoter (*i.e.* proximal 580 bp) is sufficient for basal activity of the gene (8). Through deletion analysis of the human transferrin promoter, a cell type-specific region was identified within the proximal –175 bp (8). In hepatocytes, this proximal region binds HNF (proximal regions I, P_{RI}) and c/EBP (P_{RII}) and imparts liver specificity (9). In rat Sertoli cells, the basal transcription of the human transferrin promoter is due to the TATA box region.

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The increase in transcription is most likely through the combination of factors binding to the proximal sites (PRI and PRII). Interestingly, the cAMP response element binding protein (CREB) interacts with the PRII (-100) of the human transferrin promoter (10, 11). In Sertoli cells, FSH acts primarily through the cAMP-PKA pathway (12). The regulation of the transferrin gene expression by FSH appears to be in part due to the CREB binding at PRII, however, PRII shows no homology to a consensus cAMP response element (CRE) (10, 11). Deletion of sequences upstream of the -100 bp region of the human transferrin (hTf) promoter retained cAMP responsiveness (10). This proximal 100 bp of the hTf contains the CREB binding region PRII. The effects of FSH on this proximal -100 bp promoter suggests the potential involvement of response elements upstream of this 100 bp region.

Another response element that is highly conserved between the mouse and human transferrin promoters is the E-box response element. The E-box is a consensus hexanucleotide sequence (CANNTG) and binds bHLH transcription factors as heterodimers (13). The bHLH proteins are a class of transcription factors previously shown to be involved in the transcriptional control of cell specific differentiation in a number of tissues including muscle and brain (e.g. MyoD and neuroD) (14, 15). These transcription factors have a conserved HLH domain essential for dimerization and a basic domain. The paired basic domain mediates binding to a consensus E-box (CANNTG) (16). Previously Sertoli cells have been shown to express bHLH proteins in response to FSH and PModS(S300) (17). Overexpression of Id (a negatively acting HLH protein lacking a basic domain) down-regulates the mouse transferrin promoter activation (17). The down-regulation of the mouse transferrin promoter may be directly mediated at the level of the E-box in the promoter, or it may be indirect involving intermediate early-event transcription factors.

The previous observations that additional regions of the mTf promoter other than the PRII are needed for optimal FSH actions (10, 11) and that Id can alter FSH actions (17) suggest that the actions of FSH are more complex than simply a CRE element requirement. In the present study, both E-box and CRE cis-acting DNA elements were demonstrated to be required for FSH induced activation of the mouse transferrin promoter in rat Sertoli cells. Observations suggest that bHLH proteins acting at the E-box and CREB acting at the CRE-like PRII appear to cooperate for optimal FSH induced promoter activation.

Materials and Methods

Cell preparations and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (18) with a modified procedure described by Tung *et al.* (19). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco BRL, 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 Chemical Co.). Sertoli cells were then plated under serum-free conditions in 24-well Falcon plates at 1×10^6 cells/well. Cells were maintained in a 5% CO₂ atmosphere in Ham's F-12 medium (Gibco BRL) with 0.01% BSA at 32 C. Sertoli cells were left untreated (Control) or treated with either FSH (100 ng/ml; o-FSH-16, National Pituitary Agency), dbcAMP (0.1

mm) or PModS (S300) (50 µg/ml). The PModS (S300) is a partially purified testicular paracrine factor isolated on a Sephacryl S300 column (7). These optimal concentrations of hormones and PModS (S300) have previously been shown to dramatically stimulate cultured Sertoli cell differentiated functions (19, 20). The cells were cultured under serum-free conditions for a maximum of 5 days with a media change after 48 h of culture. Cell number and density did not change during the culture in the absence or presence of the treatment (20, 21).

Plasmids

The CAT reporter plasmid (pUC8-CAT) containing -581 bp (-581 bp mTf-CAT) was generously provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA) (22). The mouse transferrin promoter used in the present study included the transcriptional initiation site of the transferrin gene which is 54 bp upstream of the start site of translation (22). The point mutations in the PRII and E-box elements of the mTf-CAT promoter were created with a QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The introduction of the desired mutation was confirmed by DNA sequencing of the reporter constructs (Fig. 1).

Transfection

Sertoli cells cultured for 48 h were transfected with a reporter gene construct by the calcium phosphate method coupled with hyper osmotic shock (10% glycerol) as previously described (23). Briefly, 1.5 µg reporter plasmid in 150 µl of transfection buffer (250 mM CaCl₂ mixed 1:1 vol/vol with 2x Hebes [28 mM NaCl, 50 mM HEPES, and 1.47 mM Na₂HPO₄, pH 7.05]) was added to each well of a 24-well plate containing 1×10^6 Sertoli cells in 1 ml of Ham's F-12 with 0.01% BSA, and incubation was performed at 32 C for 4 h. After incubation, the cells were subjected to a hyper osmotic shock. The medium was aspirated, and 1 ml of 10% glycerol in HBSS (Gibco BRL) was added. The cells were incubated for 3 min, and the wells were washed twice before fresh Ham's F-12 was added. Various treatments were subsequently added, and cells were incubated for 48 h before harvesting for CAT assays. In each experiment the transfection efficiency was monitored by transfecting the Sertoli cells by the plasmid containing β-galactosidase gene driven by a CMV promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in 25% transfection efficiency.

CAT assay

Assay of CAT activity was performed as follows: medium was removed from the wells, and the cells were washed once with PBS. One hundred microliters of cell lysis buffer (Promega Corp.) was added to each well, and incubation was carried out for 15 min at room temperature. The wells were then scraped and buffer was collected in 1.5-ml microfuge tubes. Tubes were heated to 65 C for 10 min to inactivate endogenous acetylases and then centrifuged at 12,000 × g for 10 min at 4 C to remove cell debris. An aliquot of cell extract (54 µl) was mixed with 65 µl 0.25 M Tris, pH 8.0, 25 µg n-butyryl coenzyme A (5 mg/ml; Sigma Chemical Co.), and 0.1 µCi (1 µl) of ¹⁴C-Chloramphenicol (ICN, Costa Mesa, CA) and incubated overnight at 37 C. The mixture was extracted once with 300 µl mixed xylenes and back-extracted with 100 µl of 0.25 M Tris (pH 8.0). A 200-µl aliquot of the organic phase was counted in a scintillation counter to determine the relative amount of CAT activity. The average conversion of CAT substrate for treated cells ranged between 20 and 30%. This assay was found to be linear with the protein concentration used.

Gel shift assays

Gel shift assays were performed with nuclear extracts of isolated Sertoli cells. The Sertoli cells were isolated as described above and cultured in 150 mm × 20 mm tissue culture dishes (Nunc). The cells were treated after 48 h in culture with either FSH, PModS(S300) or not treated for controls. 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.* (8). Typically 70–100 µg of protein was obtained from 10⁶-plated cells. The double-stranded DNA probes with flanking sequences used in gel retardation

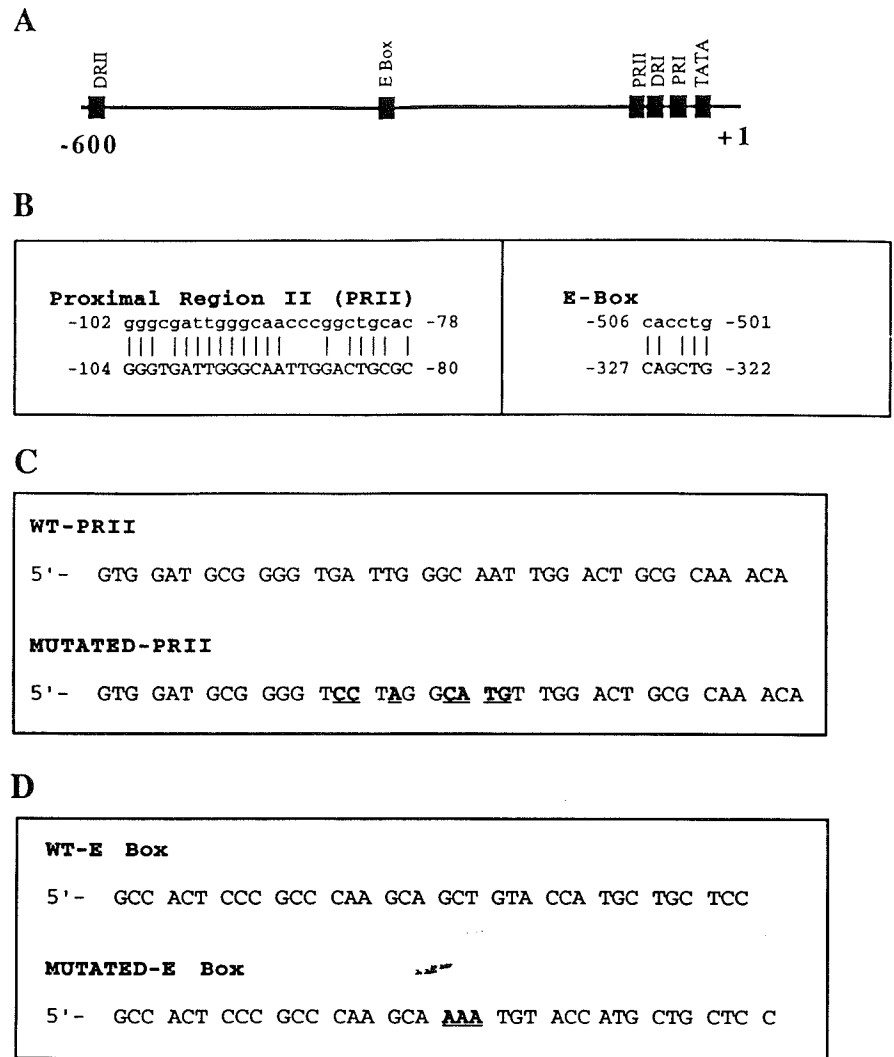


FIG. 1. A, Potential response elements in the proximal 581-bp mouse transferrin promoter. Proximal regions I (PRI) and II (PRII) and distal regions I (DRI) and (DRII) were identified by comparing the mouse transferrin promoter to the human transferrin promoter. B, Sequence comparison between human (*lower case*) and mouse (*upper case*) proximal region PRII and E-box. C, Sequence of the WT-PRII and mutated-PRII oligonucleotides used as a probe in gel shift assays and to introduce mutations in the mouse transferrin promoter. D, Sequence of the WT-E-box and mutated-E-box oligonucleotides used as a probe in gel shift assays and to introduce mutations in the mouse transferrin promoter.

assays were: E-Box: GCCCAAGCAGCTGTACCATGC; Mutated E-box: GCCCAAGCAAAATGTACCATGC; PRII: GCGGGGTGATTGGGCAATTGGACT; Mutated PRII: GCGGGTCTAGGCATGTTG GACT; CRE: GTGGATGACGTCAGGTCA. The gel retardation assay used was a modification of the protocol described by Garner and Rezvin (24). The final reaction volume of 20 μ l contained 0.5 ng (approximately 50,000 cpm) of 5'-³²P-labeled double stranded probe, 100 ng sonicated salmon sperm DNA, 2 μ g Poly dI-dC (USB), 20 μ g BSA, 20 mM HEPES, pH 8.0, 4 mM Tris, pH 7.9, 50 mM KCl, 600 μ M EDTA and EGTA, 500 μ M DTT, and 5 μ g Sertoli cell nuclear proteins. After incubation at room temperature for 20 min, 5 μ l of the reaction was electrophoretically separated on a nondenaturing 5% polyacrylamide gel in 0.5 \times TBE. The gel was dried under vacuum and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at -80 C for 16-18 h. For the competition experiments, excess unlabeled oligonucleotide (250 fold in excess than labeled probe) was added in the binding reaction.

Immunoblot procedure

A gel shift assay was performed in duplicate on the same gel using radioactive and nonradioactive PRII or E-box oligonucleotide. The gel shift using radioactive probes was dried and autoradiographed as above. Gel shift assay using nonradioactive probes on the polyacrylamide gel was electrophoretically transferred to a nitrocellulose membrane (BA85, Schleicher & Schuell, Inc.) by electrophoresis in Tris-gly-

cine buffer containing 12% methanol. The blot was then blocked in 5% nonfat milk in TBSN (50 mM Tris, pH 7.4; 150 mM NaCl, and 0.05% Nonidet P-40) and incubated with a 1:3000 dilution of antibodies to E12/E47, CREB and nonimmune serum (NIS) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 3 h. After three washes of 15 min each, the blot was hybridized with a secondary antibody (1:3000 dilution; directed against rabbit immunoglobulin G) conjugated to alkaline phosphatase for 1 h at room temperature. After 5 washes in TBSN, the immune complex was detected using the Immune-Star Chemiluminescent Protein Detection System (Bio-Rad Laboratories, Inc.). As an internal control, a gel shift was carried out using AP-1 oligo and blotted with *c-fos*, E12, USF, and SRF antibody. When AP-1 was used as a probe, a band in the blot was observed only with *c-fos* antibody (data not shown).

Statistical analysis

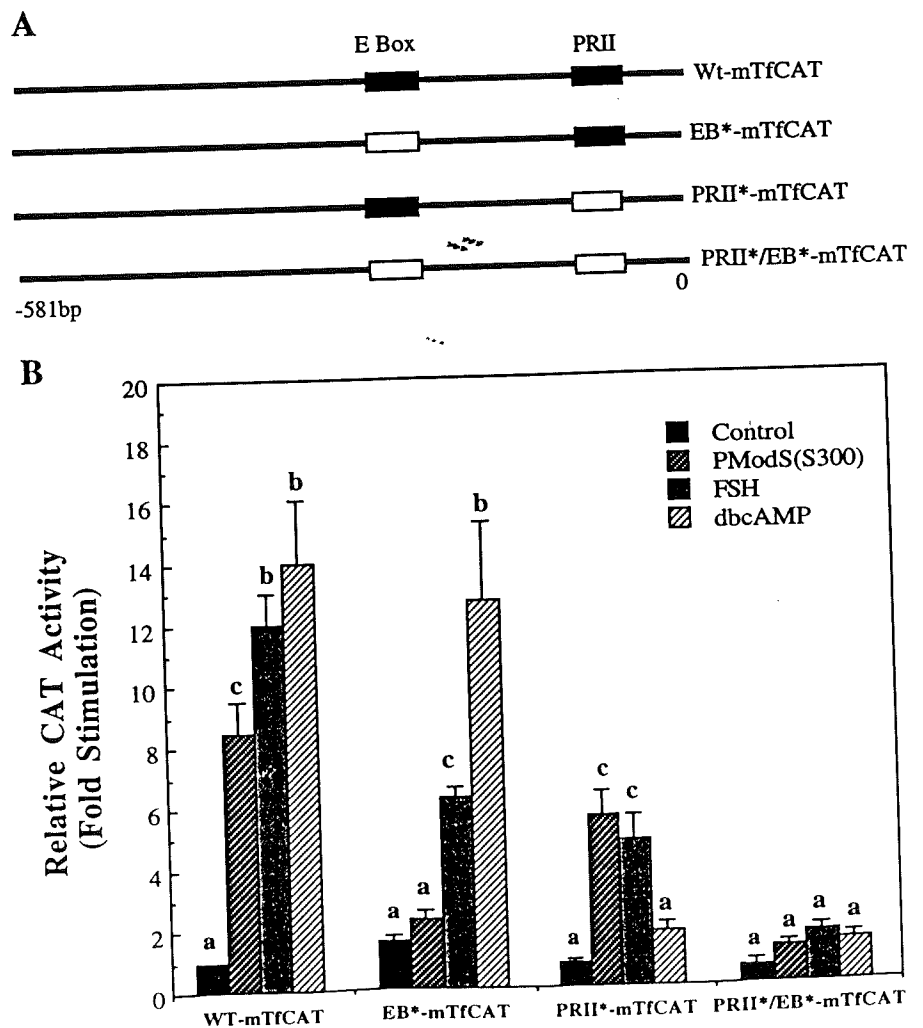
All transfection data were obtained from a minimum of three different experiments unless otherwise stated. Each data point (from treatments) was converted to a relative CAT activity (control CAT activity of WT-mTf CAT = 1) with the mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by ANOVA as indicated in the figure legends. The CAT reporter plasmids without mTf promoter was used as negative controls. In response to PModS (S300), FSH, and dcAMP the relative CAT activity of the negative control plasmid was in the range of 1.5 to 2.

Results

The hypothesis was tested that bHLH proteins and cAMP response element binding proteins regulate the transcription of the mouse transferrin promoter in Sertoli cells. A chimeric construct containing the chloromphenicol acetyltransferase (CAT) reporter driven by the proximal 581 bp of the mouse transferrin promoter (WTmTf-CAT) was transiently transfected into cultured Sertoli cells, (Fig. 1A). Sertoli cells were isolated from 20-day-old rat testis and cultured under serum free conditions. After transfection, the cells were left untreated or treated with either FSH, dbcAMP, or a partially purified bioactive preparation from a size exclusion column termed PModS(S300) for 48 h before harvesting the cells for CAT assay. As shown in Fig. 2, FSH, cAMP, and PModS(S300) induced 12-, 14- and 8-fold stimulation of the CAT activity, respectively, compared with untreated controls ($P < 0.001$). This increase in CAT activity was significantly higher than that observed by transfecting Sertoli cells with a plasmid containing only the CAT gene without any promoter, (less than 2-fold stimulation, data not shown). The increased CAT activity in response to dbcAMP suggests that the -581bp of the proximal promoter contains a cAMP response element.

Sequence analysis of the proximal promoter did not reveal the presence of a consensus cAMP response element (CRE). Therefore, in an attempt to define the regulatory sequences that mediate the cAMP response, a comparison was made with the proximal mouse and human transferrin promoters. Although the proximal human transferrin promoter also does not contain a consensus CRE site, the CREB was shown by Suire *et al.* (10, 11) to bind a region termed PRII with high affinity (10, 11). The sequence of the human PRII region is shown in Fig. 1B and has minimal similarity to the consensus CRE site (TGACGTCA). The human PRII sequence was more than 80% conserved in the mouse transferrin promoter (Fig. 1B). A gel shift assay was performed to determine whether the mouse PRII can form specific DNA protein complexes. As shown in Fig. 3, the 24-bp PRII oligonucleotide resulted in a gel shift with multiple bands when nuclear extracts from FSH and dbcAMP treated Sertoli cells were used in the binding reaction (Fig. 3). The presence of multiple retarded bands raises the possibility that PRII may bind a number of different proteins (or isoforms) with different mobility's in the native gel. Excess unlabeled PRII was added to the binding reaction to confirm that the retarded bands were specific. The excess unlabeled PRII displaced all the observed bands suggesting

FIG. 2. A, Schematic of the mutant mouse transferrin promoter constructs. Mutation in either E-box or PRII or both E-box and PRII were generated in context of the intact promoter. Solid rectangles represent the WT, and open rectangles represent the mutations in either E-box or PRII. B, Effect of E-box and PRII mutations on the activation of mouse transferrin promoter in Sertoli cells. The cultured Sertoli cells were transfected with the mutant transferrin promoter-CAT constructs and were either left untreated (Control) or treated with FSH, PModS(S300) or dbcAMP as indicated. Data are presented as relative CAT activity of WT-mTf-CAT control (WT-mTf-CAT control = 1) and is the mean \pm SEM of duplicate samples in three separate experiments. The absolute value of conversion of CAT substrate in terms of cpm (cpm) was not significantly different in untreated Sertoli cells transfected with various mutant reporter constructs. Different superscript letters above the error bars represent a statistically significant difference ($P < 0.001$).



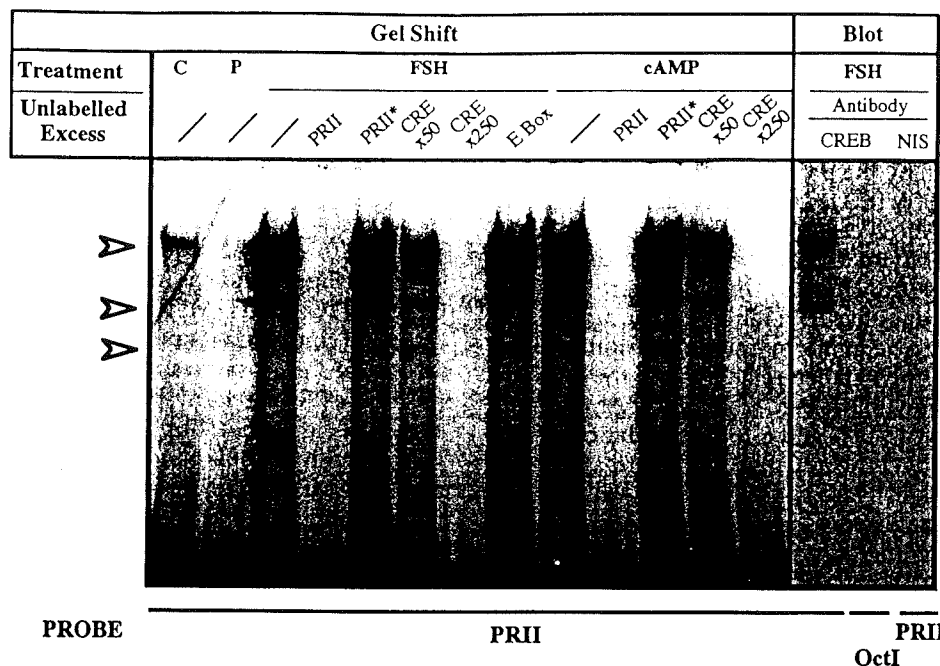


FIG. 3. The gel shift with mouse PRII oligonucleotide (WT-PRII) and nuclear extracts (5 μ g) from untreated Sertoli cells (C) or treated with FSH, PModS(S300) (P) or cAMP. Three major bands as indicated by the arrows were observed when nuclear extracts from FSH- and cAMP-treated Sertoli cell extracts were used. All of the shifted bands were specific because addition of excess unlabeled PRII but not mutated PRII oligonucleotide (PRII*) could displace them. A complete displacement was also observed when excess unlabeled consensus CRE (GTG-GATGACGTCAGGTCA) was included in the binding reaction. The panel on the right of the gel shift represents the immunoblot (Blot) as described in *Materials and Methods*. The PRII gel shift blotted onto nitrocellulose membrane was probed with antibody to CREB. Two retarded bands similar in migration to the bands I and II of the gel shift were detected indicating the presence of CREB. No band with the CREB antibody was detected when Oct 1 oligonucleotide was used as a probe or when NIS was used. Data are representative of a minimum of three different experiments.

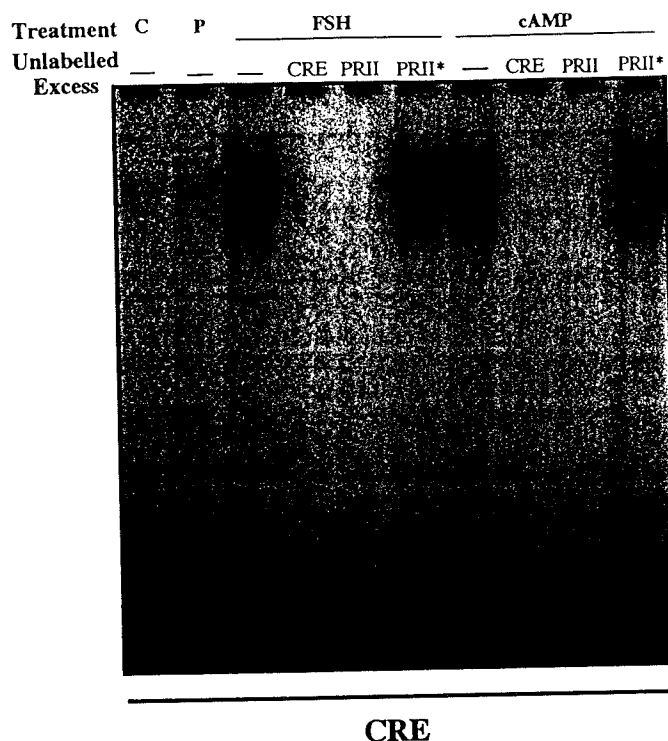
that these bands were specifically due to binding of proteins to the PRII oligonucleotide (Fig. 3). To explore the possibility that CREB may bind to PRII, excess unlabeled consensus CRE oligonucleotide was added to the binding reaction. Addition of excess CRE also displaced all three of the major bands in the gel shift reaction (Fig. 3). However, none of the bands were displaced when either E-box or Oct1 oligonucleotides were included in the binding reaction (data not shown). The gel shift was transferred onto a nitrocellulose membrane and blotted with antibody to CREB to confirm that PRII does bind CREB. As shown in Fig. 3 (blot, far right) two bands were detected with CREB antibody which correlated with the first two bands in the gel shift assay. As a control for the blot experiment, a gel shift with Oct 1 oligonucleotide was transferred onto nitrocellulose membrane and probed with CREB antibody. Failure to detect any retarded band with CREB antibody confirmed the specificity of the blot procedure and support the observation that CREB is present in the complex binding to PRII. The identity of the bottom gel shift is currently unknown and suggests that additional proteins may also bind the PRII oligonucleotide. Further experiments were performed with a consensus CRE oligonucleotide. This consensus CRE (TGACGTC) was previously shown to bind CREB (25). As shown in Fig. 4, a faint retarded complex was observed when nuclear extracts from untreated controls (C) and PModS(S300) treated Sertoli cells were used in the binding reaction. The intensity of this retarded complex was increased dramatically when nuclear extracts from either

FSH- or dbcAMP-treated cells were used. This result suggests that both these treatments increased the binding of CREB to the consensus CRE. This retarded complex was specific due to the ability of excess unlabeled CRE to completely displaced the CRE gel shift. Interestingly, addition of unlabeled PRII also completely displaced the retarded complex, confirming that PRII also bound CREB (Fig. 4). Therefore, activation of the WT-mTfCAT in response to cAMP (Fig. 2) appears to be mediated through the PRII site.

Mutations were performed to understand the contribution of PRII to the activation of the transferrin promoter. Site directed mutagenesis of the PRII site in the 581-bp minimal promoter was done to introduce specific nucleotide changes (Fig. 1C). In a gel shift reaction a mutated PRII oligonucleotide (PRII*) failed to compete the retarded bands using wild-type (WT) PRII (Fig. 3) or WT-CRE (Fig. 4) as probes. This suggests that mutations in the PRII appear to disrupt the binding of CREB. In transient transfection experiments, the mTfCAT construct with mutations in the PRII region (PRII*-mTfCAT) was stimulated only 2-fold ($P < 0.05$) in response to dbcAMP (Fig. 2). This is a significant reduction in the activity of the PRII*-mTfCAT promoter when compared with the activation of the WT-mTfCAT by dbcAMP (14-fold, $P < 0.001$). The data suggests that the stimulation of the transferrin promoter by cAMP is primarily mediated through the binding of CREB at the PRII site. However, in response to FSH the PRII*-mTfCAT retained 40% of its activity (5-fold, $P < 0.001$) compared with untreated controls but was sig-

nificantly less than the WT-mTfCAT (12-fold, $P < 0.001$) in response to FSH (Fig. 2). Comparison of the PRII*-mTfCAT response to cAMP (2-fold) and FSH (5-fold) suggested that

other response elements apart from PRII are also involved in FSH mediated activation of the transferrin promoter. Interestingly, the activity of PRII*-mTfCAT and WT-mTfCAT was similar in response to PModS (S300) activity (Fig. 2). This supports the previous hypothesis (7) and provides direct evidence that PModS (S300) activity does not act through the cAMP-PKA pathway.

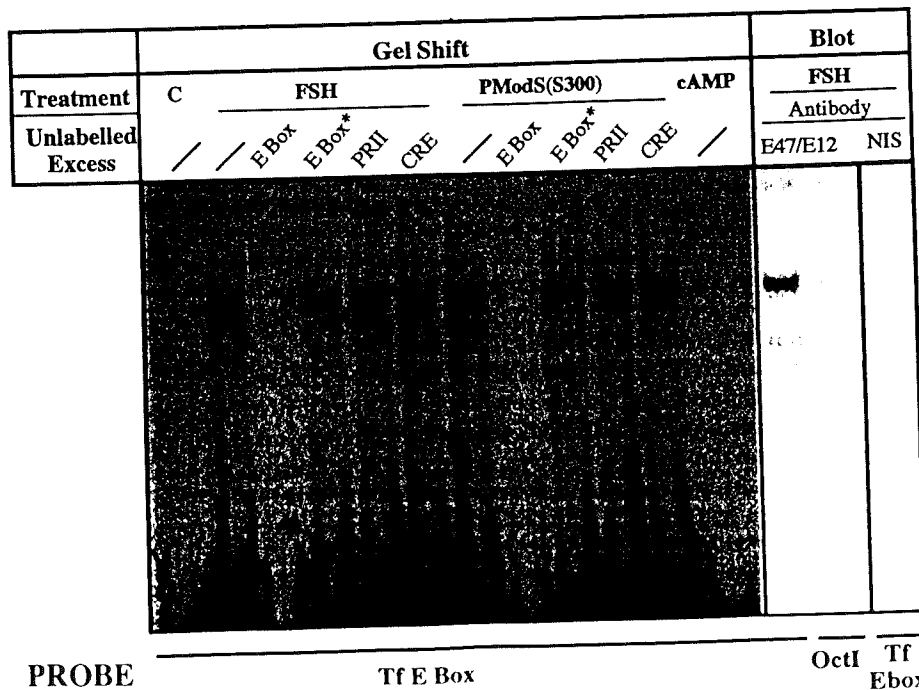


CRE

FIG. 4. Gel shift indicating the presence of specific proteins in the nuclear extracts of FSH and dbcAMP treated Sertoli cell that can bind a consensus CRE oligonucleotide (GTGGATGACGTCAAGTCA). Excess unlabeled WT-PRII oligonucleotide (PRII) and mutated PRII oligonucleotide (PRII*) are indicated. Treatments are indicated: C, control; P, PModS(S300); FSH; and dcAMP (cAMP). Data are representative of three different experiments.

Previous reports demonstrate that overexpression of the negatively acting HLH protein Id down-regulates the mouse transferrin promoter activity. Both PModS(S300) activity and FSH were also shown to induce the bHLH protein activity in Sertoli cells (17). This and the observation that a consensus E-box is present in both mouse and human proximal transferrin promoters (Fig. 1A) suggested a potential role for this E-box in regulating the activity of the mouse transferrin promoter. Gel shift experiments were performed to confirm that E-box binding proteins can bind to the transferrin E-box. As shown in Fig. 5, a specific retarded band was observed when the mouse transferrin E-box (Tf E-box) oligonucleotide was used in the binding reaction with nuclear extracts from Sertoli cells treated with FSH and PModS(S300). A faint retarded band comparable to untreated controls was also observed when nuclear extracts from dbcAMP treated Sertoli cells were used in the binding reaction. Observations suggest that cAMP may not induce the activity or expression of bHLH proteins (Fig. 5). A gel shift immunoblot experiment was performed as previously described to identify the protein(s) binding to the E-box in response to FSH. The gel shift with nuclear extracts from FSH-treated Sertoli cells was transferred onto a nitrocellulose membrane and blotted with antibody to the ubiquitously expressed bHLH proteins E12/E47. The presence of a single band comparable to the retarded band in the gel shift assay confirmed that the product of the E2A gene E47/E12 is part of the complex binding to the transferrin E-box. Site directed mutations in the core

FIG. 5. Gel shift with transferrin E-box oligonucleotide (WT-E-box). Sertoli cells were untreated control (C) or treated with FSH, PModS(S300), or cAMP. Excess unlabeled oligonucleotides used were E-box, mutated E-box*, PRII, and CRE. Data are representative of three different experiments. The panel on the right of the gel shift represents the immunoblot (Blot) as described in *Materials and Methods*. The E-box gel shift blotted onto nitrocellulose membrane was probed with antibody to E12/E47. A single retarded band similar in migration to the gel shift band on the left indicates the presence of E12/E47. No band with E12/E47 antibody was detected when Oct 1 oligonucleotide was used as a probe or when NIS was used.



nucleotides of the E-box in the 581-bp proximal promoter were performed to investigate the role of the E-box in regulating the mouse transferrin promoter (Fig. 1C). As shown in Fig. 5, the failure of the mutated E-box oligo to displace the retarded band confirmed that the mutations disrupt the binding of E-box proteins.

Cultured Sertoli cells were transfected with the 581-bp transferrin promoter reporter construct containing the E-box mutations (EB*-mTfCAT). The cells were then treated with either FSH, dbcAMP, PModS (S300), or were untreated (C). The dbcAMP treatment resulted in a 12-fold ($P < 0.001$) stimulation of the EB*-mTfCAT construct (Fig. 2) and was comparable to the WT promoter. Therefore, mutations in the E-box had no effect on the activation of the transferrin promoter by dbcAMP. This is consistent with the gel shift data shown in Fig. 5 indicating the absence of a retarded band with dbcAMP. The activity of EB*-mTfCAT construct in response to FSH was significantly reduced (6-fold) compared with the WT promoter ($P < 0.001$) (Fig. 2). Only 2-fold stimulation (nonsignificant) was observed when the EB*-mTfCAT construct was stimulated with PModS(S300). The transfection data using EB*-mTfCAT suggests that the action of FSH on the mouse transferrin promoter are mediated in part through the E-box and not exclusively through the cAMP-PKA-mediated binding of CREB to the P_{RII} site. This is supported by the observation that the dbcAMP treatment had no effect on the EB-mTfCAT promoter, but the activity of P_{RII}-mTfCAT promoter was completely inhibited. The testicular paracrine activity PModS(S300) seems to primarily regulate the activity of the mouse transferrin promoter through the binding of bHLH proteins to the E-box.

A complete inhibition in the mouse transferrin promoter activation in response to PModS(S300) and dbcAMP was observed when either E-box or P_{RII} were mutated respectively. Neither of these mutations alone completely abolished FSH actions on the mouse transferrin promoter. The proposal was made that both these response elements are required for optimal FSH induced activation of the promoter. To test this hypothesis simultaneous mutations were performed in both the E-box and P_{RII} of the 581-bp minimal promoter. The Sertoli cells were transiently transfected with the mutated E-box and P_{RII} sites in the promoter (EB*-P_{RII}*-mTfCAT) and treated with FSH. As shown in Fig. 2, the CAT activity of the double mutant EB*-P_{RII}*-mTf construct was comparable to untreated controls. This observation suggests that intact E-box and P_{RII} sites are required for maximal stimulation of the mouse transferrin promoter by FSH (Fig. 6). The potential general role of both the E-box and CRE-like elements in mediating FSH actions was examined by investigating the presence of both of the elements in the published promoter sequences of a number of Sertoli cell genes. As shown in Table 1, a consensus E-box sequence was present in promoters of all the sequences examined. Although the complete 25 bp of the P_{RII} region was not completely conserved, parts of the region were highly conserved. The P_{RII}-like domain may have the potential to regulate the PKA mediated activation of these promoters. This is supported by the observation that a consensus CRE site was absent from the promoters analyzed with the exception of the inhibin promoter (Table 1). Although this suggests the potential that

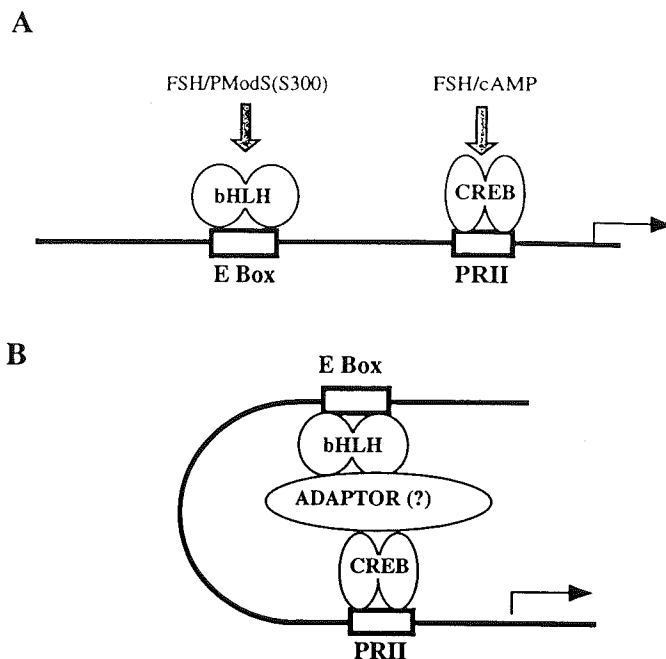


FIG. 6. Schematic showing the possible mechanisms by which CREB or related cAMP-inducible proteins and bHLH proteins activate the transferrin promoter. A) The activation of the mouse transferrin promoter by FSH may be due to the binding of bHLH and CREB like proteins to E-box and P_{RII} respectively. Binding of bHLH proteins to the E-box in response to PModS(S300) may be sufficient for promoter activation. B, Proposed mechanism by which bHLH and CREB related proteins may interact. An optimal promoter activation in response to FSH may require the interaction between bHLH and CREB-like proteins through a common protein (Adaptor).

both these elements may be required for FSH actions, more direct studies with each specific promoter will be required.

Discussion

Transferrin is an iron binding protein involved in transporting iron to cells and is required for cell proliferation, differentiation, and metabolism (26, 27). The liver is the primary source of transferrin in serum (28), but several other extra-hepatic tissues like Sertoli cells (4), brain oligodendrocytes (29), choroid plexus (30), and mammary gland (31) also produce transferrin. Serum levels of iron regulate transferrin expression in the liver (2). In other tissues, transferrin expression is independent of serum iron levels and is under the control of various hormones and other stimuli (1). Therefore, transferrin is an example of a gene that is expressed in more than one cell type and involves diverse transcriptional control mechanisms regulating its expression. Multiple cis-acting elements like P_{RI}, P_{RII}, D_{RI}, D_{RII}, and CR are present in the 5'-flanking region of the human transferrin gene (8, 9) (Fig. 1). These elements can be recognized by *trans*-acting factors such as C/EBP, CREB, HNF, and COUP Tf (8, 9, 10, 11, 32). A combinatorial effect of several such *trans*-acting factors with multiple cis-acting sequences may provide tissue specific expression of the transferrin gene. In the current study, such cis-acting elements and *trans*-acting factors involved in regulating Sertoli cell expression of the mouse transferrin gene were investigated. Transferrin is secreted by

TABLE 1. Identification of potential E box, PRII, and CRE response elements in the proximal (-600 bp) promoter of Sertoli cell genes

Sertoli gene	Species	Acc. No.	Ebox CAATGG	PRII	CRE TGACGTCA
Transferrin	Human	M15673	-506 CACCTG	-102 GGGCAATTGGCAACCCGGCTGCAC	None
	Mouse	AF027386	-327 CAGCTG	-104 GGGTCAATTGGCAATTGGACTGCGC	None
	Rat	M19993	-72 CATGTG	-----CCTGGCTGCA-	None
			-165 CATCTG	-----GGGCAATTCTGCTGCA-	None
MIS	Mouse	U09208	-119 CAGCTG	-----GGGCCACCCG-----	None
		M87570	-225 CAGGTG	-----	None
			-124 CACGTG	-----	None
FSHR	Mouse	AF045163	-253 CAGGTG	-----CAGGCTTAC-----	-123 TGATGTCA
			-130 CATCTG	-----	None
Inhibin	Rat	L15617	-257 CAAGTG	-----	None
	Rat				

Bold letters represent conserved bases. ABP, Androgen binding protein; FSHR, FSH receptor; MIS, Müllerien-inhibiting substance; AR, androgen receptor. The GenBank accession number used to obtain the sequence is given.

differentiated Sertoli cells and understanding the factors involved in regulating the transferrin gene expression provides insight into Sertoli cell differentiation.

Sertoli cell function is regulated primarily by the gonadotropin FSH. The FSH receptor is coupled to the PKA pathway (33). FSH treatment rapidly increases intracellular cAMP levels in the Sertoli cells (10). Many of the actions of FSH in Sertoli cells can be mimicked by the cAMP analog dbcAMP. The cAMP-PKA pathway regulates transcription through the phosphorylation of serine 133 of the 43-kDa CREB that binds to the 8-bp palindromic sequence (TGACGTCA) CRE site present in the promoters of a number of genes (34, 35). The absence of a consensus CRE site in the mouse transferrin promoter (mTf), as well as from the promoters of other Sertoli cell genes, Table 1, suggests that either CREB or other members of its family can bind sites other than a consensus CRE. Alternatively, the effects of FSH/cAMP are mediated through intermediate transcription factors which are responsive to FSH/cAMP induction. Interestingly, the PRII site in the human transferrin promoter binds CREB at a nonconsensus CRE site (10, 11). The observation that mutations in the PRII site of the human transferrin promoter can down-regulate cAMP-induced stimulation (10) suggests that dimers of the CREB family of bZIP proteins are able to target DNA sequences different from the consensus 8-bp CRE palindrome. The presence of a nearly conserved PRII site in the mouse transferrin promoter suggests that this PRII site is a potential candidate in regulating FSH and cAMP-induced mouse transferrin gene expression. In contrast to the human PRII sequence, the mouse PRII sequence contains the CRE half site (CGTCA). Another response element that is more than 90% conserved in both human and mouse PRII is the C/EBP binding site (ANNGGCAATT). In addition to CREB, the PRII of the human transferrin promoter also can bind two additional unknown proteins SP-A and SP-D in rat Sertoli cells (9). It is possible that transcription factors similar to SP-A and SP-D may also bind PRII of the mouse transferrin promoter used in the present study and impart some tissue specificity. Our observations suggest that the PRII of the mouse transferrin promoter also binds CREB. The presence of multiple bands in the gel shift using PRII oligonucleotide as a probe (Fig. 3) suggests that multiple transcription factors can bind to PRII. However, a complete displacement of all the retarded bands with excess unlabeled consensus CRE oligonucleotide indicates that these other unknown transcription factors appear to be dependent on the binding of CREB or its isoforms to PRII.

The contribution of the PRII region of the mouse transferrin promoter in the actions of FSH was investigated by examining the activity of mTf promoter with specific mutations in the PRII region in the mTf-CAT plasmids transfected into Sertoli cells. This mutation resulted in a nearly complete loss of responsiveness to cAMP and appears to be due to the loss of binding of CREB which was suggested by the gel shift data using mutated PRII (PRII*). The mutation in the PRII had no effect on the PModS(S300) activation of the mTf promoter. This confirms previous observations (7) and provides additional evidence that the actions of PModS(S300) activity are not mediated through the cAMP pathway.

Interestingly, FSH was still able to stimulate (5-fold) the

PRII* mTf CAT plasmid. If the induction of the mTf promoter were exclusively mediated through the cAMP pathway, a complete loss in promoter activation as seen with dbcAMP was anticipated. The lack of a complete loss of responsiveness implies the role of other cis acting elements and *trans*-acting factors in FSH mediated induction of the mTf promoter. One such *trans*-acting factor may be *c-fos*. The translation of *c-fos* is required for FSH- and PModS(S300)-induced transferrin promoter activation and expression of the gene (29). The activation of *c-fos* by FSH and PModS(S300) primarily seems to be at the level of the serum response element (SRE) (36). However, a consensus CRE site is also present in the proximal *c-fos* promoter. The *c-fos* together with *c-jun* as a dimer can bind to a potential AP-1 site present in the promoters of a number of genes to activate transcription (37). The absence of an AP-1 site in the proximal mouse and human transferrin promoters again suggests that intermediate transcription factor(s) under the control of *c-fos* may regulate the activity of the transferrin promoter. Another possibility is that a common cis acting element may control the activity of both the *c-fos* and transferrin promoters. One of the common cis-elements identified by comparison of the mouse transferrin promoter with the proximal promoter of *c-fos* was an E-box element. E-box (consensus CANNTG) elements are a motif to which the bHLH class of transcription factors bind (13). These proteins are involved in cell-specific transcriptional control in a number of tissues including muscle and brain (14, 15). The bHLH proteins have a conserved HLH domain essential for dimerization of different bHLH proteins as well as a basic domain that mediates binding to an E-box (16).

An E-box is present in the proximal *c-fos* promoter (-303) and mouse transferrin promoter (-327, CAGCTG). Interestingly an E-box sequence is also present in the human transferrin promoter (-506, CACCTG). The presence of an E-box suggests that bHLH proteins may regulate the transcriptional activity of these promoters. Previously we have shown that overexpression of Id (a negatively acting HLH protein lacking a basic DNA binding domain) down-regulates the mTf-CAT (17) and *c-fos* (unpublished observation) reporter construct in response to FSH and PModS. This down-regulation by Id implies that bHLH proteins either directly regulate the mouse transferrin promoter CAT activity by binding to the E-box or mediate activation through *c-fos*. Mutations in the E-box were performed to directly establish the role of the E-box in the regulation of the mouse transferrin promoter. As observed with the PRII mutations, the E-box mutations resulted in only a 6-fold stimulation of the transferrin promoter in response to FSH. The mutated E-box had no effect on cAMP induction and was comparable to the WT promoter. This transfection data are also supported by the gel shift experiments in that a retarded E-box band was observed with FSH but not with nuclear extracts from cAMP treated Sertoli cells. These observations support the role of an E-box in the regulation of the transferrin promoter by FSH and suggest involvement of multiple cis-acting element and *trans*-acting factors such as bHLH proteins and possibly CREB. Comparison of the response of EB*-mTfCAT to FSH and cAMP suggests that binding of bHLH proteins to the E-box is present for FSH, but not cAMP. FSH signaling events

independent of the cAMP-PKA pathway may regulate the binding of bHLH proteins to the E-box. Recently, it was shown that an E-box response element is present in the proximal (-200 bp) promoter region of the regulatory subunit RII β of the cAMP dependent PKA (38). The functional significance of the E-box in the regulation of the RII β promoter activity is not known. It is possible that a Sertoli cell specific response to FSH, but not cAMP, may require the cell specific expression and binding of bHLH proteins to the RII β E-box. The presence of the E2A gene product E12 or E47 in the retarded band with an E-box as a probe was demonstrated. E12 and E47 are ubiquitously expressed bHLH proteins that generally form heterodimers with cell specific bHLH protein to activate transcription. The possibility exists that E12/E47 form heterodimers with a yet unknown Sertoli cell specific bHLH protein.

In summary, the current study provides direct evidence for the first time that the presence of both the E-box and the PRII in the transferrin promoter is required for FSH to optimally activate the mTf promoter. The presence of PRII is sufficient if the stimulation is mediated primarily through the cAMP-PKA pathway. In contrast, the effect of the testicular paracrine factor PModS(S300) activity on the promoter is mediated primarily through the E-box response element (Fig. 6A). Observations suggest that at least two different pathways are involved in regulating the activity of the transferrin gene in Sertoli cells. This is interesting considering the phenotype recently reported for the FSH β -subunit knockout mice (39). Although fertility was maintained in FSH β null mutation male mice, the testis size was significantly smaller. This appears to be due to decreased spermatogenesis and/or Sertoli cell numbers, suggesting FSH has a role in maintaining optimal testicular function. Combined observations suggest that multiple factors involving diverse signal transduction and transcription pathways appear to regulate Sertoli cell gene expression. As shown in Fig. 6B, it is proposed that bHLH proteins may interact with CREB or CREB-like proteins binding to the PRII. This interaction may be direct or mediated through an adapter-type protein. This adapter protein can be similar to CBP, which has recently been shown to bind bHLH proteins, as well as CREB (40, 41). The complete loss in the activity of the promoter in response to FSH with both E-box and PRII mutations suggests that both these response elements are required for maximal stimulation of the transferrin promoter. How these response elements and cis-acting factors interact remains to be elucidated. The observation that similar elements (*i.e.* E-box and PRII) are present in other major Sertoli cell genes (Table 1) suggests this may be a general phenomena for FSH activation of Sertoli cell transcription. More direct experiments with those various promoters will be required to test this hypothesis.

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