BIOLOGY OF REPRODUCTION **65,** 568–574 (2001)

Role of the Transcriptional Coactivator CBP/p300 in Linking Basic Helix-Loop-Helix and CREB Responses for Follicle-Stimulating Hormone-Mediated Activation of the Transferrin Promoter in Sertoli Cells

Jaideep Chaudhary[2] and Michael K. Skinner[1]

Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-4231

ABSTRACT

Sertoli cells are the epithelial cells responsible for the onset of pubertal development and the 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 regarding the regulation of Sertoli cell differentiation. The optimal activation of the mouse transferrin promoter (mTf) by FSH requires the synergistic actions of the cAMP response element-binding protein (CREB) binding to the cAMP response element-like proximal region II (PRII) and the basic helix-loop-helix (bHLH) binding to the E-box. Proximal region II alone is sufficient for cAMP-mediated activation. The proximity of the PRII and E-box (220 base pairs apart) suggests the possibility of interaction between CREB and bHLH proteins. Such an interaction can be mediated by transcriptional integrators such as CREB-binding protein (CBP) and/or p300 and may stabilize the binding of *trans*-acting factors to their respective *cis*-elements. Such an interaction may also provide a mechanism for cell-specific promoter activation. The hypothesis tested in this study was that CBP/p300 is required for the synergistic activation of the transferrin promoter involving PRII and E-box through the formation of a ternary complex. In the Sertoli cells, both CBP and p300 proteins are expressed. The effect of CBP/p300 on transferrin promoter activation and, hence, Sertoli cell function was studied by using antisense oligonucleotides (AS-oligo). In the presence of CBP/p300 AS-oligo, activity of the FSH-induced mTf-chloramphenicol acetyl transferase (CAT) was significantly lower as compared to the respective controls. Interestingly, AS-oligo had no effect on cAMP-induced activation of the transferrin promoter reporter construct (mTf-CAT). Mutations in the E-box (EB*) significantly reduced the FSH response. The presence of AS-oligo had no further effect on the FSH-mediated activation of the EB*-mTf-CAT construct but reduced cAMP-mediated activation. Mutations in the CRE-like PRII (PRII*) also significantly reduced the FSH response. Activation of the PRII*-mTf-CAT in response to cAMP was completely abolished. The presence of AS-oligo had no further effect on the FSH- or cAMP-mediated activation of the PRII*-mTf-CAT construct. In Sertoli cells, CBP/p300 was coimmunoprecipitated with CREB and the bHLH protein E47. These observations suggest that CBP/p300 appears to be involved in regulating FSH-mediated activation of the transferrin promoter by linking bHLH and CREB activities.

cyclic adenosine monophosphate, follicle-stimulating hormone, gene regulation, mechanisms of hormone action, Sertoli cells

[1]Correspondence. FAX: 509 335 2176; e-mail: skinner@mail.wsu.edu
[2]Current address: Ataigin Technologies, Inc., 101 Theory, Irvine, CA 92612.

Received: 19 January 2001.
First decision: 21 February 2001.
Accepted: 4 April 2001.

ISSN: 0006-3363. http://www.biolreprod.org

INTRODUCTION

Sertoli cells are the testicular epithelial cells that form the seminiferous tubule and provide the cytoarchitectural support and microenvironment for developing germ cells [1, 2]. This microenvironment is created through the expression of many different gene products [3]. The molecular mechanisms underlying Sertoli cell-specific gene expression are poorly understood. The transferrin gene provides a model to elucidate the regulatory mechanisms controlling the expression of a gene in different cell types [4]. Transferrin is a major secretory product of differentiated Sertoli cells and is postulated to transport iron to the developing germ cells sequestered by the blood-testis barrier [5, 6]. In contrast to the liver [7], the transferrin gene expression in Sertoli cells is not regulated by iron but, instead, by hormones such as FSH [8–10]. The gonadotropin FSH is considered to be one of the endocrine components required for the regulation of Sertoli cell function [1, 11, 12]. The actions of FSH on Sertoli cells involve the cAMP protein kinase A pathway [13, 14]. Promoter analysis of most Sertoli cell genes indicated the absence of a consensus cAMP response element (CRE) site [15]. The regulation of the human [8] and mouse [15] transferrin gene expression in rat Sertoli cells by FSH appears to be, in part, due to CRE-binding protein (CREB) binding at a CRE-like element proximal region II (PRII). Deletion of sequences upstream of the proximal 100-base pair (bp) region of the human transferrin promoter retained cAMP response, but not FSH responsiveness [8]. One of the response elements that is required to retain FSH, but not cAMP, responsiveness in the mouse transferrin promoter (mTf) is an E-box response element [15]. The E-box is a consensus hexanucleotide sequence (CANNTG) and is the binding site for basic helix-loop-helix (bHLH) transcription factor heterodimers [16]. The bHLH proteins are a class of transcription factors involved in the transcriptional control of cell-specific differentiation in a number of tissues, including muscle [17] and brain [18] (e.g., MyoD and neuroD). These transcription factors have a conserved helix-loop-helix domain that is essential for dimerization and a basic domain [19]. The paired basic domain mediates binding to a consensus E-box (CANNTG) DNA sequence [20]. Previously, Sertoli cells have been shown to express bHLH proteins in response to FSH [21].

The actions of FSH on Sertoli cells are not only mediated by phosphorylation of CREB and its subsequent binding to PRII but, also, by mechanisms that activate bHLH proteins, suggesting a synergistic action of these pathways [15]. The proximity of the E-box and PRII responsive *cis*-elements in the transferrin promoter (220 bp apart) [15] suggests that both CREB and bHLH proteins may influence transferrin promoter activation through direct protein interactions mediated by adapter proteins. One adapter protein

that is known to interact with both bHLH and CREB is CREB-binding protein (CBP) [22] and its homologue p300 [23].

Both CBP and p300 are highly homologous nuclear proteins originally cloned for their ability to interact with phosphorylated CREB [22] and with the adenovirus E1A protein [23], respectively. Both CBP and p300 are thought to function as crucial links between diverse signal transduction pathways and to play an essential role in the cellular processes of growth and differentiation [24–26]. Several protein motifs in CBP and p300, such as a bromodomain, a KIX domain, and three regions rich in Cys/His residues (C/H domains), are well conserved in CBP/p300 species ranging from *Drosophila* to mammals. The CBP/p300 consists of flexible modules that can accommodate interactions with multiple activators of transcription and that can integrate signals derived from multiple pathways. These modules serve as binding sites for sequence-specific transcription factors and other components that regulate gene expression. The CBP/p300 interactions with transcription factors include the bHLH myogenic proteins; CREB; the oncogene product Myb; the retinoic acid, estrogen, glucocorticoid, and thyroid hormone receptors; AP1 activity; and components of the basal transcription machinery, such as transcription factor IIB (TFIIB) and TATA-binding protein [24–26]. These observations suggest that CBP/p300 might constitute a physical nexus between enhancer binding proteins and components of the basal transcription machinery. In addition, CBP/p300 may disrupt repressive chromatin structures through its intrinsic or associated histone acetyl transferase activity [27, 28].

The regulation of Sertoli cell gene expression in response to FSH appears to involve multiple signal transduction pathways. Therefore, coactivators may function as signal integrators by coordinating complex signal transduction events at the transcriptional level. The present study demonstrates that in Sertoli cells, the synergistic actions of two transcription factors, the bHLH proteins and CREB, on the transferrin promoter are integrated by the coactivators CBP/p300.

MATERIALS AND METHODS

Isolation of Sertoli Cells

Sertoli cells were isolated from the testis of 20-day-old rats according to a previously described procedure [29, 30] with some modifications. The isolated Sertoli cells were more than 98% pure and plated under serum-free conditions. Cells were maintained in a 5% CO_2 atmosphere in Ham F-12 medium (Gibco, Rockville, MD) with 0.01% BSA at 32°C. Sertoli cells were treated with either FSH (100 ng/ml; o-FSH-16, National Pituitary Agency, Torrance, CA), dibutryl cAMP (dbcAMP; 100 μM), 10% bovine calf serum, or vehicle alone (Ham F-12, control). These optimal concentrations of FSH and dbcAMP have previously been shown to optimally stimulate cultured Sertoli cell differentiated functions [30, 31]. The cells were cultured for a maximum of 5 days, with a media change and treatment after 48 h of culture. Cell number, purity, and viability did not change during the culture in the absence or presence of treatment [31, 32].

Western Blotting and Immunoprecipitation

Sertoli cells were cultured in 150-mm plates and treated with FSH and dbcAMP as described above. After 72 h of treatment, the cells were washed twice with Hanks balanced salt solution and lysed with 1 ml of M-PER lysis buffer (Pierce, Rockford, IL) supplemented with miniprotean protease inhibitor cocktail (Boehringer, Indianapolis, IN) at 4°C for 30 min. Lysates were centrifuged at 10 000 × *g* for 30 min at 4°C and the supernatants collected. The protein concentration in the supernatants was estimated using a Bradford assay (Bio-Rad, Hercules, CA). Approximately 50–150 μg of protein in SDS sample loading buffer were boiled for 5 min and electrophoresed on a 5% mini SDS gel (Bio-Rad). The protein was subsequently transferred onto nitrocellulose membrane and probed with specific rabbit polyclonal antibodies to CBP and p300 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specific antigen-antibody complex was visualized using an alkaline phosphatase chemiluminescent detection kit (Bio-Rad).

For immunoprecipitation, the Sertoli cells were cultured in 150-mm plates and treated with FSH and dbcAMP as described above. The cells were washed with phosphate-buffered saline and lysed at 4°C for 30 min with 1 ml of M-PER lysis buffer (Pierce) supplemented with miniprotean protease inhibitor cocktail (Boehringer). Lysates were centrifuged at 10 000 × *g* for 30 min at 4°C and the supernatants collected. Each supernatant was incubated with 5 μl of normal rabbit IgG and 50 μl of packed protein A-agarose beads (Santa Cruz Biotechnology) at 4°C for 1 h. The complex formed was removed by centrifugation at 500 × *g* for 5 min at 4°C. The supernatant was then incubated with either 5 μl of normal rabbit IgG, anti-rabbit E12/E47, or anti-rabbit CREB antibodies for 4 h at 4°C. Immunoprecipitates were collected on protein A-agarose beads and washed four times with M-PER lysis buffer and two times in TSA buffer (10 mM Tris [pH 8.0] and 140 mM NaCl). The precipitate was dissolved in 30 μl of SDS sample buffer, boiled for 5 min, and subjected to electrophoresis on a 5% mini SDS gel. The fractionated proteins were transferred onto nitrocellulose membranes. The membranes were then blotted with mouse monoclonal antibody to CBP/p300 (Chemicon, Temecula, CA). The specific antigen-antibody complexes were detected with anti-mouse IgG conjugated to horse radish peroxidase.

Plasmids and Antisense Oligonucleotides

The chloramphenicol acetyl transferase (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). The mTf 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 [33]. The mTf-CAT reporter plasmids with either EB or PRII mutations were constructed as described previously [15]. The antisense oligonucleotides to CBP and p300 were designed to incorporate 15 bases, 5′ to and including the translational initiation site. Because the rat CBP and p300 were not available, a consensus was developed using the available mouse and human CBP and p300 mRNA sequences. The following amino acid sequences were used to design antisense oligonucleotides:

CBP: MAENLL (GenBank: human, U85962; mouse, S66385) (CPB 5′-CAG CAA GTT CTC GGC CAT-3′)

P300: MAENVV (GenBank: human, NM_001429; mouse [protein], AAB31182) (p300 5′-CAC CAC ATT CTC GGC CAT-3′)

The scrambled oligonucleotides were generated by the GCG software analysis package (Tampa, FL) using the respective CBP (CBP-scr. 5′-CGA TGC GTC ATC GAC CAT-3′) and p300 (p300-scr. 5′-GCA TCC GTA CAA CCT TCC-3′) antisense oligonucleotides. The antisense and the scrambled oligonucleotides (showing no substantial homology to any known genes, as determined through a BLASTn search) were synthesized from commercial sources using phosphorothioate modification.

Transfections

Sertoli cells cultured in 24-well plates at a density of 10^6 cells/well for 48 h were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described [15]. Briefly, 1.5 μg of reporter plasmid in 150 μl of transfection buffer (250 mM $CaCl_2$ mixed 1:1 [v:v] with 2× Hebes] 28 mM NaCl, 50 mM Hepes, and 1.47 mM Na_2HPO_4 at pH 7.05]) was added to each well of a 24-well plate containing 10^6 Sertoli cells in 1 ml of Ham F-12 with 0.01% BSA, and incubation was performed at 32°C for 4 h. After incubation, the cells were subjected to a hyperosmotic shock. The medium was aspirated, and 1 ml of 10% glycerol in Hanks balanced salt solution (Gibco) was added. The cells were incubated for 3 min, and the wells were washed twice before fresh Ham F-12 was added. The transfected Sertoli cells were treated with 4 μM of either the antisense or scrambled oligonucleotides immediately after transfection. Various treatments were added to the cells 2 h after the addition of antisense oligonucleotides. The cells were retreated with the oligonucleotides every 12 h for a total of 72 h. In each experiment, the transfection efficiency was monitored by transfecting the Sertoli cells with a plasmid containing the β-galactosidase gene driven by a CMV promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in approximately 25% transfection efficiency.

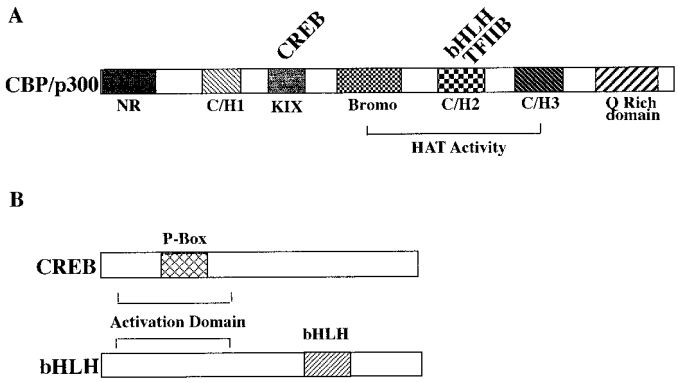

FIG. 1. **A**) Schematic showing the domains of CBP and p300 known to bind various transcription factors. The KIX domain binds CREB, and the C/H2 domain binds TFIIB and the bHLH family of transcription factors. **B**) The P-box within the activation domain of CREB binds the KIX domain in CBP/p300, and the activation domain of bHLH family of transcription factors binds the C/H2 domain of CBP/p300.

CAT Assay

Assay of CAT activity was performed to monitor promoter construct activity in the cells. Medium was removed from the wells, and the cells were washed once with phosphate-buffered saline. One hundred microliters of the cell lysis buffer (Promega, Madison, WI) were added to each well and incubated 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 of 0.25 M Tris (pH 8.0), 25 μg of n-butyryl coenzyme A (5 mg/ml; Sigma, St. Louis, MO), and 0.1 μCi (1 μl) of ^{14}C-chloramphenicol (ICN, Costa Mesa, CA) and incubated overnight at 37°C. The mixture was extracted once with 300 μl of 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.

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 = 1) with the mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by an analysis of variance (ANOVA) with the SAS statistical package (Cary, NC) as indicated in the figure legends. The CAT reporter plasmid without mTf promoter was used as a negative control. In response to FSH and dbcAMP, the relative CAT activity of the negative control plasmid was in the range of 1.5 to 2.

RESULTS

Both CBP and p300 bind a number of different transcription factors through highly conserved domains. As shown in Figure 1, the domains of CBP/p300 that bind bHLH and CREB transcription factors are distinct. This suggests the possibility of integrating these *trans*-acting factors given the proper spatial proximity of the respective *cis*-elements. The CREB binds to the KIX domain, whereas the bHLH proteins bind to the cystine-rich C/H2 domain within the histone acetyl transferase activity region of CBP/p300 (Fig. 1A). The activation domains of CREB and bHLH are shown in Figure 1B.

The hypothesis that CBP/p300 may act as a transcriptional coactivator in FSH-mediated transferrin promoter activation was tested. Experiments were initially performed to show that CBP/p300 is present in Sertoli cells. Sertoli cell proteins were fractionated on 5% SDS gels, transferred

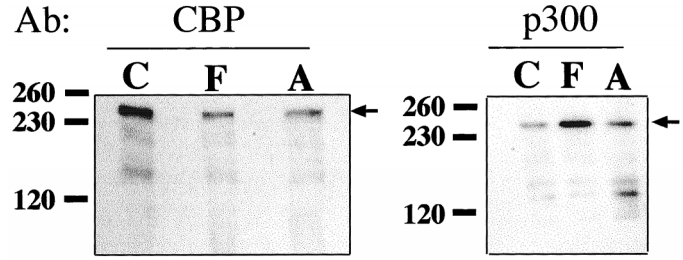

FIG. 2. Western blot analysis of CBP and p300 in Sertoli cells. Total Sertoli cell proteins were fractionated and transferred onto nitrocellulose membranes. The membranes were then blotted with specific polyclonal antibodies (Ab) to either CBP or p300. The CBP and p300 proteins (shown by the arrowhead at ~240 kDa) were detected using second antibody conjugated to alkaline phosphatase. The blot is a representative of at least three different experiments performed on different Sertoli cell protein preparations. A, dbcAMP; C, control; F, FSH.

to nitrocellulose membranes, and probed with polyclonal antibodies specific to either CBP or p300. As shown in Figure 2, both CBP and p300 are detected in Sertoli cell proteins. When data were normalized for protein loading and actin immunostaining, no hormone regulation was observed (data not shown). This experiment suggests that both CBP and p300 are constitutively expressed by Sertoli cells (Fig. 2).

An antisense approach was used to evaluate the possible functional role of CBP/p300 in the regulation of transferrin promoter activation. A chimeric construct containing the CAT reporter driven by the proximal 581 bp of the mTf (WTmTf-CAT) was transiently transfected into cultured Sertoli cells (Fig. 3). 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 or dbcAMP for 72 h before harvesting for CAT assay. As shown in Figure 3, FSH and dbcAMP induced 8- and 14-fold stimulation, respectively, of the wild-type transferrin promoter (WT-Tf) CAT activity as compared to 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 cDNA without any promoter (less than twofold stimulation, data not shown). To evaluate the role of CBP and p300 on FSH- and dbcAMP-mediated WT-Tf promoter activation, the Sertoli cells transfected with WT-Tf promoter CAT construct were also treated with an antisense to either CBP or p300. When used alone, neither of the antisense oligonucleotides had an effect on WT-Tf promoter activation (data not shown) in the range of concentrations used (2–10 μM).

Both CBP and p300 perform similar functions [34], and potential compensation may explain the lack of any effect from either CBP or p300 antisense oligonucleotides alone on the transferrin promoter activation. Therefore, experiments were performed in which antisense to both CBP and p300 were added together and their influence on transferrin promoter activation evaluated. In the presence of the antisense oligonucleotides to both CBP/p300 (2 μM each), the FSH-stimulated WT-Tf CAT activity was significantly decreased (Fig. 3). Interestingly, in the presence of the antisense oligonucleotides to both CBP/p300, the dbcAMP-stimulated WT-Tf CAT activity decreased approximately 20% ($P < 0.05$) (Fig. 3). No decrease in the WT-Tf CAT activity was observed in the presence of scrambled CBP/p300 oligonucleotides, suggesting that the effects of the CBP/p300 antisense oligonucleotides on FSH- or dbcAMP-

stimulated WT-Tf CAT activity were specific (Fig. 3). The presence of the antisense oligonucleotides had no effect on the CAT activity of nonstimulated (i.e., control) Sertoli cells. The inhibitory effect of the CBP/p300 antisense oligonucleotides on the transferrin promoter activation was further confirmed by treating the Sertoli cells transfected with the thymidine kinase minimal promoter (tk)-CAT promoter construct with CBP/p300 antisense oligonucleotides. The presence of CBP/p300 antisense oligonucleotides had no effect on either FSH- or dbcAMP-stimulated activation of the tk-CAT promoter construct (data not shown). Therefore, the presence of CBP/p300 appears to be necessary for FSH and, to a lesser degree, cAMP to induce Sertoli cell functions (i.e., transferrin promoter activation).

To address this hypothesis that CBP forms a ternary complex with Sertoli cell bHLH proteins and CREB, Sertoli cells were transfected with transferrin promoter constructs with mutations in either the E-box (EB*) or the PRII site (PRII*). The activity of EB*-mTf-CAT construct in response to FSH was significantly reduced (45%) as compared to the WT-Tf promoter ($P < 0.001$) (Fig. 3). The dbcAMP treatment resulted in a 12-fold ($P < 0.001$) stimulation of the EB*-mTf-CAT construct (Fig. 3) and was comparable to the wild-type promoter. Therefore, EB* had no effect on the activation of the transferrin promoter by dbcAMP. Consistent with previous results [15], the mTf-CAT construct with mutations in the PRII region (PRII*-mTf-CAT) was stimulated only threefold ($P < 0.05$) in response to dbcAMP (Fig. 3). This is a significant reduction in the activity of the promoter when compared to the activation of the WT-mTf-CAT by dbcAMP (14-fold, $P < 0.001$). The data suggest that 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*-mTf-CAT retained 50% of its activity (fourfold, $P < 0.001$) as compared to untreated controls but was significantly less than the FSH-induced WT-mTf-CAT activity (eightfold, $P < 0.001$). No further effect was observed when Sertoli cells transfected with either EB*-mTf-CAT or PRII*-mTf-CAT were treated with antisense oligonucleotides to CBP/p300 and challenged with either dbcAMP or FSH. The absence of any effect of the antisense oligonucleotides following FSH treatment on the EB*-mTf-CAT and PRII*-mTf-CAT promoter constructs suggests that an intact E-box and PRII site may be required for CBP/p300 to mediate its effect on the transferrin promoter. An interesting observation was the small effect of CBP/p300 antisense oligonucleotides on dbcAMP-stimulated activation of EB*-mTf-CAT promoter. These data suggest that CBP/p300 may have a role in the activation of the transferrin promoter through the cAMP-protein kinase A pathway.

Immunoprecipitations were performed to demonstrate that CBP/p300 interacts with CREB and Sertoli cell bHLH proteins like E47. Total Sertoli cell proteins following FSH treatment were extracted and subjected to immunoprecipitations with polyclonal antibodies to either normal rabbit IgG (i.e., control) or rabbit polyclonal antibodies specific to either E47 or CREB. The same quantity of nuclear extract protein was used in the different immunoprecipitations. The immunoprecipitates were resolved on 5% SDS-polyacrylamide gels and the proteins transferred onto nitrocellulose membranes. The membranes were then blotted with CBP/p300 mouse monoclonal antibodies, and the immune complexes were detected using anti-mouse IgG conjugated to alkaline phosphatase. The mouse monoclonal antibodies were used to minimize cross-reactivity with the rabbit polyclonal antibodies that were used to perform immunoprecipitations. As shown in Figure 4, a distinct CBP/p300 band was observed when immunoprecipitations were performed with either E47 or CREB antibodies, but not with normal rabbit IgG. These results demonstrate that in Sertoli cells, CBP/p300 can associate with CREB and bHLH proteins like E47. Combined data suggest that the synergistic actions of bHLH and CREB on the transferrin promoter are in part mediated through the coactivators CBP/p300 (Fig. 5).

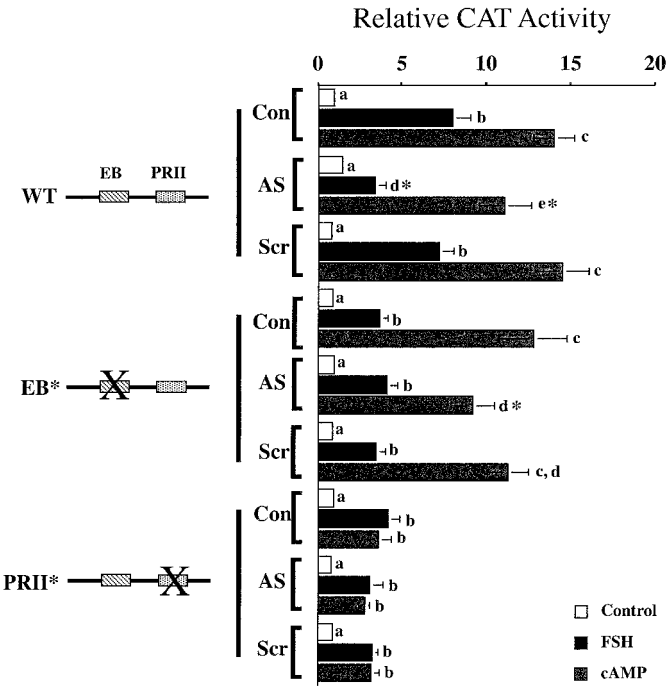

FIG. 3. Effect of a combination of antisense oligonucleotides to CBP and p300 on the transferrin promoter-CAT activity. The cultured Sertoli cells were transfected with the wild-type, proximal 600-bp mTf-CAT construct (WT) or transferrin CAT construct with mutations in either the E-box (EB*) or PRII (PRII*) response elements. Immediately following the transfection, CBP (2 μM) and p300 (2 μM) antisense (AS) or scrambled (Scr) phosphorothioate-modified oligonucleotides were added and compared to untreated cultures (Con). The cells were challenged with FSH, dbcAMP, or vehicle alone (Control) 2 h after the addition of oligonucleotides. The oligonucleotides were subsequently added every 12 h until the cells were harvested for CAT assay (72 h). Data are presented as relative CAT activity of mTf-CAT control (without any treatment) set to 1 and are the mean ± SEM of triplicate samples in three separate experiments. Different superscript letters above the error bars represent a statistically significant difference ($P < 0.05$) with ANOVA analysis within each treatment group.

DISCUSSION

The cAMP-binding protein CREB and members of the bHLH class of transcriptional regulators play a significant role in Sertoli cell differentiation and development [8, 14, 15, 21]. In Sertoli cells, the phosphorylation of CREB at ser133 is primarily mediated by FSH-dependent activation of the protein kinase A pathway [14]. Although the role of bHLH phosphorylation is unknown, several kinases have been described that phosphorylate E47 in vitro [38–40]. The activation of Sertoli cell function and differentiation involves integration of diverse signaling pathways at the level of transcription factors. Such an integration may involve transcriptional coactivators. Few molecules have been demonstrated to act as transcriptional coactivators in eukaryotic cell systems. In the current study, the role of two coactivators, CBP and p300, in regulating FSH-depen-

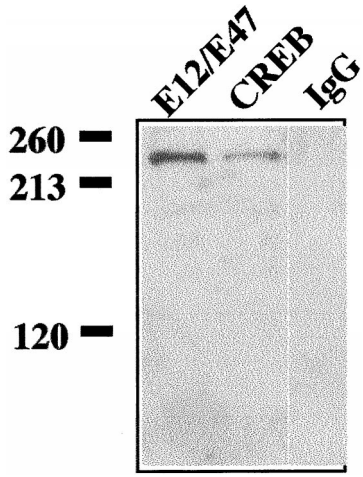

FIG. 4. Coimmunoprecipitation of CBP and p300 with E47 and CREB. Total Sertoli cell proteins were incubated with nonimmune serum (IgG) or polyclonal antibodies to either E47 or CREB. Immunoprecipitates were fractionated and transferred onto nitrocellulose membranes. Western blot of the membranes used mouse monoclonal antibody cross-reactive with both CBP and p300. The CBP/p300 was detected when Sertoli cell proteins were immunoprecipitated with E47 and CREB, but not with nonimmune IgG. The blot is a representative of at least three different Western blots performed on Sertoli cell proteins isolated at different times.

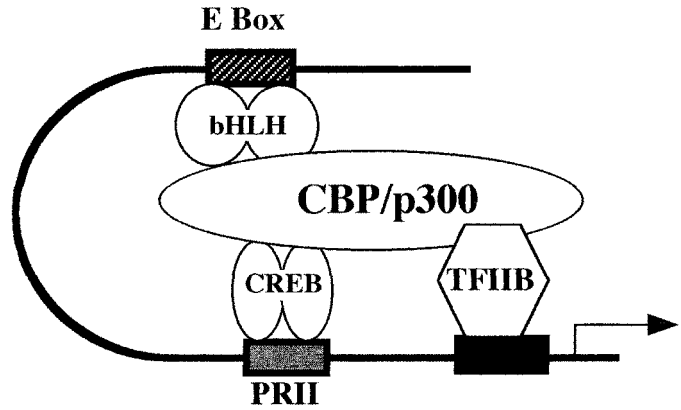

FIG. 5. Schematic showing the possible mechanism by which CREB or related cAMP-inducible proteins and bHLH proteins activate the transferrin promoter. An optimal promoter activation in response to FSH may require the interaction between bHLH and CREB-like proteins through transcriptional coactivators such as CBP and p300. The activation of the mTf by FSH may be due to the formation of ternary complex involving the binding of bHLH and CREB-like proteins to E-box and PRII, respectively, and transcriptional coactivators such as CBP and p300. The CBP/p300 may also bind to the components of the basal transcriptional machinery such as TFIIB. The intrinsic histone acetyl transferase activity of CBP/p300 and the spatial orientation of PRII and E-box around histones may cause DNA bending. The bending and relaxation of the chromatin structure may facilitate an organization that, in turn, facilitates the interaction of bHLH proteins, CREB, and CBP/p300.

dent transferrin promoter activation in Sertoli cells were investigated. The CBP and p300 were selected due to the following characteristics: 1) they share significant sequence homology, 2) they are capable of coactivating the same target genes, and 3) they both bind CREB and bHLH proteins known to regulate transferrin promoter activity in Sertoli cells.

Results of previous studies have suggested a synergistic role of both the E-box and the PRII in FSH-mediated transferrin promoter activation in Sertoli cells [15]. The presence of PRII appears to be sufficient for stimulation by cAMP, which is mediated primarily through the cAMP-protein kinase A pathway. These observations suggest that multiple factors involving diverse signal transduction and transcription pathways appear to regulate Sertoli cell gene expression. It is speculated, given the proximity of these two *cis*-elements [15], that the bHLH proteins that bind to E-box elements may interact with CREB or CREB-like proteins binding to the PRII. This interaction may be direct or mediated through an adapter-type protein, which can also stabilize the binding of the corresponding *trans*-acting factors. The formation of such a ternary complex may also provide a mechanism to regulate cell-specific transcription. The adapter protein CBP can bind both bHLH proteins and CREB [34–37]. In the present study, the hypothesis was tested that CBP/p300 is involved in integrating the *trans*-acting factors involved in FSH regulation of the transferrin promoter in Sertoli cells.

Both CBP and p300 protein expression was observed in Sertoli cells. As shown in other cell types, CBP protein expression appears to be constitutive. To address the functional significance of CBP/p300 in regulating Sertoli cell gene expression, an antisense approach was used. An alternate approach considered was the overexpression of wild-type and mutant forms of CBP/p300. However, the advantage of using antisense is that it provides a system in which the cell is essentially devoid of the protein. Because CBP/p300 was present in the cells independent of hormone stimulation, the antisense approach was selected.

The decrease in the FSH-mediated transferrin promoter activity in the presence of antisense oligonucleotides to both CBP and p300 suggests that CBP/p300 are involved in the regulation of the transferrin promoter. The functions of both CBP and p300 appear to be compensatory in Sertoli cells, because the use of antisense oligonucleotides individually had no effect on the transferrin promoter. Compensatory functions for both CBP and p300 have been previously observed [34–37], and to our knowledge, this is the first confirmation of this effect in Sertoli cells. The FSH receptor is coupled to the protein kinase A pathway, and FSH treatment rapidly increases intracellular cAMP levels in Sertoli cells [14]. Many of the actions of FSH in Sertoli cells can be mimicked by the cAMP analogue dbcAMP [14]. Previously, it has been shown that butyrate has no effect on Sertoli cell differentiated functions, such as transferrin expression. The inhibitory effect of antisense oligonucleotides on the FSH-mediated transferrin promoter activation suggests that FSH actions may involve multiple signal transduction pathways that are dependent on the activity of CBP/p300. The regulation of the transferrin promoter by cAMP alone appears not to be as dependent on CBP/p300.

The PRII site on the human transferrin promoter binds CREB with strong association and dissociation kinetics [41]. It was proposed that such a strong binding of CREB to PRII might be stabilized by transcriptional coactivators such as CBP [41]. However, overexpression of CBP in Sertoli cells did not potentiate the basal and cAMP-stimulated activity of CREB of the −100 to +39 Tf-CAT construct containing PRII [41]. This observation supports the data presented in this study that the cAMP-stimulated activation of the transferrin promoter is primarily independent of CBP/p300.

The known *cis*-elements in the mTf that mediate the effect of FSH in Sertoli cells are the E-box and PRII sites [15]. Both these sites have a synergistic effect in regulating

the activity of the transferrin promoter in response to FSH. Surprisingly, the antisense oligonucleotides to CBP/p300 had no effect on the transferrin promoter construct with mutations in either the E-box or PRII elements. Mutations in the E-box or PRII disrupt the binding of the Sertoli cell bHLH proteins and CREB, respectively. It is speculated that in the absence of such binding, CBP/p300 may not be able to bind and/or influence these *trans*-acting factors individually. Alternatively, if the effect of CBP/p300 were mediated through response elements other than the E-box and PRII, then a reduction in the CAT activity by antisense oligonucleotides to CBP/p300 with either E-box or PRII mutations would have been apparent. The absence of such an affect on either of the mutated promoter constructs suggests that CBP/p300 may mediate its effect through intact E-box and PRII sites, possibly by forming a ternary complex involving the bHLH and CREB transcription factors. The immunoprecipitation data demonstrate that Sertoli cell CBP can associate with CREB and bHLH transcription factors such as E47. Interestingly, the domains of CBP that associate with bHLH proteins and CREB are distinct [35–37], which raises the possibility that formation of such a ternary complex is possible. In vitro binding assays are now needed to show that all three proteins are components of such a complex. Another observation that supports the formation of such a ternary complex is that CBP/p300 may stabilize the binding of the respective *trans*-acting factors to their corresponding *cis*-elements [26]. The formation of such a ternary complex involving SF-1, CREB, and CBP was recently reported on the α-inhibin promoter [42].

The CBP and p300 possess histone acetyl transferase (HAT) activity [27, 28]. Targeted histone acetylation is believed to neutralize the positive charge of the histone tails and to relax the interactions between histones and negatively charged DNA, making the gene promoters accessible. The CBP and p300 are the only known HATs that are capable of acetylating all four core histones [43, 44]. The E-box and PRII are approximately 220 bp apart, which corresponds to one turn of the DNA around the nucleosome complex. Such close proximity and the intrinsic HAT activity of CBP/p300 further support the hypothesis that CBP/p300 may form a ternary complex involving bHLH and CREB proteins (Fig. 5).

In conclusion, the present study demonstrates that CBP and p300 are expressed in Sertoli cells, and that CBP/p300 is required for optimal FSH stimulation of the transferrin promoter. Based on the effect of antisense oligonucleotides on wild-type and E-box and PRII mutants of the transferrin promoter, it is speculated CBP/p300 may form a ternary complex involving bHLH and CREB transcription factors (Fig. 5). The present data suggest that the formation of a ternary complex by CBP/p300 may facilitate integration of transcriptional regulators such as bHLH proteins and CREB in Sertoli cells. This is postulated to result in a positive cross-talk between transcription factors and the signal transduction pathways in Sertoli cells. The possibility that CBP/p300 may also integrate the basal transcription machinery to the bHLH and CREB transcription factors is also likely, as shown in Figure 5.

REFERENCES

1. Jegou B. The Sertoli cell. Bailliere's Clin Endocrinol Metab 1992; 6: 273–311.
2. Skinner MK. Cell–cell interactions in the testis. Endocr Rev 1991; 12: 45–77.
3. Griswold MD. Protein secretions of Sertoli cells. Int Rev Cytol 1998; 110:133–156.
4. Zakin MM. Regulation of transferrin gene expression. FASEB J 1992; 6:3253–3258.
5. Skinner MK, Schlitz SM, Anthony CT. Regulation of Sertoli cell differentiated function: testicular transferrin and androgen-binding protein expression. Endocrinology 1989; 124:3015–3024.
6. Forti G, Barni T, Vannelli BG, Balboni GC, Orlando C, Serio M. Sertoli cell proteins in the human seminiferous tubule. J Steroid Biochem 1989; 32:135–144.
7. Idzerda RL, Huebers H, Finch CA, McKnight GS. Rat transferrin gene expression: tissue-specific regulation by iron deficiency. Proc Natl Acad Sci U S A 1986; 83:3723–3727.
8. Suire S, Fontaine I, Guillou F. Follicle stimulating hormone (FSH) stimulates transferrin gene transcription in rat Sertoli cells: cis and trans-acting elements involved in FSH action via cyclic adenosine 3′,5′-monophosphate on the transferrin gene. Mol Endocrinol 1995; 9:756–766.
9. Huggenvik J, Sylvester SR, Griswold MD. Control of transferrin mRNA synthesis in Sertoli cells. Ann N Y Acad Sci 1984; 438:1–7.
10. Skinner MK, Griswold MD. Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol Reprod 1982; 27:211–221.
11. Griswold MD. The central role of Sertoli cells in spermatogenesis [see comments]. Semin Cell Dev Biol 1998; 9:411–416.
12. Sanborn BM, Caston LA, Buzek SW, Ussuf KK. Hormonal regulation of Sertoli cell function. Adv Exp Med Biol 1987; 219:561–588.
13. Hansson V, Skalhegg BS, Tasken K. Cyclic-AMP-dependent protein kinase (PKA) in testicular cells. Cell specific expression, differential regulation and targeting of subunits of PKA. J Steroid Biochem Mol Biol 2000; 73:81–92.
14. Walker WH, Fucci L, Habener JF. Expression of the gene encoding transcription factor cyclic adenosine 3′,5′-monophosphate (cAMP) response element-binding protein (CREB): regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells. Endocrinology 1995; 136:3534–3545.
15. Chaudhary J, Skinner MK. E-box and cyclic adenosine monophosphate response elements are both required for follicle-stimulating hormone-induced transferrin promoter activation in Sertoli cells. Endocrinology 1999; 140:1262–1271.
16. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 1989; 58:537–544.
17. Weintraub H, Dwarki VJ, Verma I, Davis R, Hollenberg S, Snider L, Lassar A, Tapscott SJ. Muscle-specific transcriptional activation by MyoD. Genes Dev 1991; 5:1377–1386.
18. Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 1995; 268:836–844.
19. Murre C, McCaw PS, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 1989; 56:777–783.
20. Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 1991; 66:305–315.
21. Chaudhary J, Cupp AS, Skinner MK. Role of basic-helix-loop-helix transcription factors in Sertoli cell differentiation: identification of an E-box response element in the transferrin promoter. Endocrinology 1997; 138:667–675.
22. Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 1993; 365:855–859.
23. Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev 1994; 8:869–884.
24. Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. Genes Dev 2000; 14:1553–1577.
25. Goldman PS, Tran VK, Goodman RH. The multifunctional role of the co-activator CBP in transcriptional regulation. Recent Prog Horm Res 1997; 52:103–120.
26. Giordano A, Avantaggiati ML. p300 And CBP: partners for life and death. J Cell Physiol 1999; 181:218–230.
27. Ait-Si-Ali S, Polesskaya A, Filleur S, Ferreira R, Duquet A, Robin P,

Vervish A, Trouche D, Cabon F, Harel-Bellan A. CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. Oncogene 2000; 19:2430–2437.
28. Magnaghi-Jaulin L, Ait-Si-Ali S, Harel-Bellan A. Histone acetylation in signal transduction by growth regulatory signals [see comments]. Semin Cell Dev Biol 1999; 10:197–203.
29. Dorrington JH, Roller NF, Fritz IB. Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Mol Cell Endocrinol 1975; 3:57–70.
30. Tung PS, Skinner MK, Fritz IB. Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell-enriched cultures. Biol Reprod 1984; 30:199–211.
31. Skinner MK, Fetterolf PM, Anthony CT. Purification of a paracrine factor, P-Mod-S, produced by testicular peritubular cells that modulates Sertoli cell function. J Biol Chem 1988; 263:2884–2890.
32. Skinner MK, Fritz IB. Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. Proc Natl Acad Sci U S A 1985; 82:114–118.
33. Idzerda RL, Behringer RR, Theisen M, Huggenvik JI, McKnight GS, Brinster RL. Expression from the transferrin gene promoter in transgenic mice. Mol Cell Biol 1989; 9:5154–5162.
34. Partanen A, Motoyama J, Hui CC. Developmentally regulated expression of the transcriptional cofactors/histone acetyltransferases CBP and p300 during mouse embryogenesis. Int J Dev Biol 1999; 43:487–494.
35. Eckner R, Yao TP, Oldread E, Livingston DM. Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. Genes Dev 1996; 10:2478–2490.
36. Sharma A, Moore M, Marcora E, Lee JE, Qiu Y, Samaras S, Stein R. The NeuroD1/BETA2 sequences essential for insulin gene transcription colocalize with those necessary for neurogenesis and p300/CREB binding protein binding. Mol Cell Biol 1999; 19:704–713.
37. De Cesare D, Sassone-Corsi P. Transcriptional regulation by cyclic AMP-responsive factors. Prog Nucleic Acid Res Mol Biol 2000; 64: 343–369.
38. Johnson SE, Wang X, Hardy S, Taparowsky EJ, Konieczny SF. Casein kinase II increases the transcriptional activities of MRF4 and MyoD independently of their direct phosphorylation. Mol Cell Biol 1996; 16:1604–1613.
39. Sloan SR, Shen CP, McCarrick-Walmsley R, Kadesch T. Phosphorylation of E47 as a potential determinant of B-cell-specific activity. Mol Cell Biol 1996; 16:6900–6908.
40. Shen CP, Kadesch T. B-cell-specific DNA binding by an E47 homodimer. Mol Cell Biol 1995; 15:4518–4524.
41. Suire S, Maurel MC, Guillou F. Follitropin action on the transferrin gene in Sertoli cells is mediated by cAMP-responsive-element-binding-protein and antagonized by chicken ovalbumin-upstream-promoter-transcription factor. Eur J Biochem 1996; 239:52–60.
42. Ito M, Park Y, Weck J, Mayo KE, Jameson JL. Synergistic activation of the inhibin alpha-promoter by steroidogenic factor-1 and cyclic adenosine 3′,5′-monophosphate. Mol Endocrinol 2000; 14:66–81.
43. Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. Nature 1996; 384:641–643.
44. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996; 87:953–959.