BIOLOGY OF REPRODUCTION **54,** 692–699 (1996)

Transcriptional Regulation of Sertoli Cell Differentiation by Follicle-Stimulating Hormone at the Level of the c-*fos* and Transferrin Promoters[1]

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ABSTRACT

One of the primary endocrine hormones that influence the onset of Sertoli cell differentiation at puberty and help maintain differentiation in the adult testis is FSH. FSH can modulate the majority of Sertoli cell differentiated functions, including stimulation of the iron-binding protein transferrin. Previous studies have shown that FSH alters the levels of cAMP and the immediate early gene c-*fos*. The current study was designed to investigate the transcriptional regulation of Sertoli cell differentiation by examining the actions of FSH on the promoter of the immediate early gene c-*fos* and the promoter of the downstream differentiated function gene transferrin. The regulation of c-*fos* by FSH was investigated with various chloramphenicol acetyltransferase (CAT) constructs containing segments of the c-*fos* promoter, such as the serum response element (SRE), cAMP response element (CRE), and AP1/phorbol ester/TPA response element (TRE), that were transfected into cultured Sertoli cells. Observations indicate that FSH can stimulate all three response elements, as well as a whole c-*fos* promoter construct. Interestingly, FSH was found to have a more dramatic effect on the SRE-CAT than a cAMP analog, suggesting a difference in the actions of the two agents. Gel mobility shift assays were performed to confirm the reporter gene results. Nuclear extracts of FSH-stimulated Sertoli cells caused a labeled AP1 oligonucleotide to form a DNA/protein complex (i.e., gel shift), indicating activation of the c-*fos* gene and binding of the c-*fos*/*jun* complex. Nuclear extracts from both FSH- and cAMP-stimulated Sertoli cells promoted similar gel shifts with SRE and CRE oligonucleotides. This observation supports the reporter gene data in indicating that FSH can influence both the SRE and CRE. A gel mobility shift assay was also performed with an oligonucleotide containing the 5′-flanking ETS domain of the SRE (ETS-SRE) that allows the formation of a ternary complex. FSH-stimulated Sertoli cell nuclear extracts were found to promote a unique ETS-SRE gel shift not present in cAMP-stimulated cells. The observations imply that FSH actions on the SRE are in part distinct from the actions of cAMP. Transferrin gene expression was examined to study the downstream regulation of Sertoli cell differentiation. CAT constructs containing deletion mutants of a 3-kb mouse transferrin promoter were used. When transfected into Sertoli cells, the 581-bp transferrin minimal promoter, previously shown to contain a CRE, had a significant response to cAMP and FSH. The 1.6-, 2.6-, and 3-kb transferrin promoter constructs also responded to FSH and cAMP to the same extent as, or to a lesser extent than, the 581-bp minimal promoter. Interestingly, the actions of FSH on the 581-bp minimal transferrin promoter were more dramatic than those of cAMP. The importance of FSH-induced c-*fos* in the regulation of transferrin expression was demonstrated in the current study when a c-*fos* antisense oligonucleotide was found to partially inhibit (50%) the ability of FSH to induce the expression of a transferrin promoter (CAT) construct. Therefore, FSH appears to act through multiple transcriptional activation pathways. The first involves cAMP and the CRE at both early-event genes (e.g., c-*fos*) and downstream genes (e.g., transferrin). It is likely that other pathways involve alternate signal transduction events (e.g., calcium mobilization) and promoter response elements (e.g., SRE). These multiple pathways may act in a compensatory manner to assure the ability of FSH to influence Sertoli cell differentiation and/or in a synergistic manner to amplify FSH actions.

INTRODUCTION

Within the testis, the Sertoli cell is the epithelial cell that forms the seminiferous tubule and provides the physical support and microenvironment required for the developing germinal cells. Therefore, Sertoli cells have an essential role in the control and maintenance of the process of spermatogenesis. Cell fate for the Sertoli cell is determined in the embryonic gonad at the time of testis determination. The majority of Sertoli cell differentiated functions appear at the onset of puberty and increase to optimal levels upon the completion of puberty in the adult animal. The expression of a large number of specific gene products establishes a differentiated Sertoli cell. The induction and increase in the expression of these genes during puberty reflect a progression of Sertoli cell differentiation. Optimal expression of these genes in the adult reflects the maintenance of Sertoli cell differentiation. An example of such a Sertoli cell gene product is the expression of the iron-binding protein transferrin. Transferrin gene expression increases during puberty and is maximally expressed in the adult Sertoli cell. Therefore, the current study utilized transferrin expression as a marker of the progression and maintenance of Sertoli cell differentiation.

An endocrine hormone that has a specific role in influencing Sertoli cell differentiation is FSH [1, 2]. Androgens are also essential for maintaining optimal Sertoli function in the adult. FSH acts at a G-protein-linked receptor specifically localized on Sertoli cells [3, 4]. Binding of FSH to its receptor activates adenyl cyclase and increases the levels of cAMP [1, 5–7]. Many of the actions of FSH on Sertoli cells can be mimicked by cAMP [2, 8]. FSH has been shown to influence Sertoli cell gene expression through the cAMP response element (CRE) on specific promoters [9]. Although

Accepted November 9, 1995.

Received May 26, 1995.

[1]This research was supported by an NIH grant (HD 20583) to M.K.S. and a Rockefeller Postdoctoral Fellowship to J.C.

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cAMP is the major signal transduction pathway regulated by FSH [1], FSH also appears to modulate other signal transduction pathways. FSH has been shown to influence calcium mobilization [10–12]. The cross talk of various signal transduction pathways in Sertoli cells has been previously investigated and established [11, 13–16]. Therefore, FSH appears to have the ability to influence Sertoli cell function and differentiation through multiple signal transduction pathways. FSH also appears to influence immediate-early gene responses [17, 18]. One of the first early-event genes found to be influenced by FSH was c-*fos* [19, 20]. The fos protein is a proto-oncogene that is transiently stimulated by a variety of mitogens and differentiation factors. FSH also promotes a transient increase in c-*fos* in Sertoli cells [19]. The role c-*fos* has in mediating the actions of FSH on Sertoli cell differentiated function was investigated in the current study.

FSH influences the majority of Sertoli cell differentiated functions previously identified [21]. A major Sertoli cell differentiated function is synthesis of the iron-binding protein, transferrin [22]. Transferrin is postulated to transport iron to developing germ cells sequestered by the blood-testis barrier of the seminiferous tubule [21, 22]. Transferrin expression is stimulated by FSH [8, 23, 24] and has been used as a marker of Sertoli cell differentiation during pubertal development [25]. The mouse and human transferrin promoters have been partially characterized with minimal proximal promoters that contain a CRE and other response elements [26]. However, the hormonal regulation of the transferrin promoter has not been previously investigated. The current study was designed to investigate the transcriptional regulation of Sertoli cell differentiation by FSH using transferrin as a downstream gene and c-*fos* as an early-event gene.

MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion [27] with a modified procedure described by Tung et al. [28]. Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco-Bethesda Research Laboratories, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma Chemical Company, St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were then plated under serum-free conditions in 24-well Falcon plates at 1×10^6 cells per well. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco-BRL) with 0.1% BSA at 32°C. Sertoli cells were left untreated (control) or were treated with either FSH (100 ng/ml; ovine [o]-FSH-16, NHPP, Rockville, MD) or dibutyryl cAMP (dbcAMP; 0.1 mM; Sigma). These concentrations of FSH and cAMP have previously been shown to maximally stimulate Sertoli cells.

Reporter Gene Constructs

*The c-*fos *promoter.* The chloramphenicol acetyltransferase (CAT) reporter plasmid (PBL-CAT2) with thymidine kinase minimal promoter [29] was used to make various c-*fos* promoter constructs as follows. The CRE-CAT had a 120-bp 5′-flanking sequence from the transcriptional start site of the human c-*fos* promoter with 5′-TGACGTTT-3′ sequence at −60 bp. The TPA/activating protein (AP1) response element (TRE: 5′-TGCGTCA-3′)-, the serum response element (SRE: 5′-CAGGATGTCCAAATTAGGACATC-3′)-, and the sis-inducible element (SIE: 5′-CATTTCCCGTAAATC-3′)-CAT reporter plasmids were constructed by cloning synthetic annealed oligonucleotides into PBL-CAT2 plasmid upstream of TK minimal promoter. The entire 400-bp c-*fos* promoter CAT construct was generously provided by Dr. Jeff Holt (Vanderbilt University, Nashville, TN) [30].

Tf promoter. The CAT reporter plasmid (PUC8-CAT) containing −581 bp (−581 bp mTf-CAT) and the human growth hormone reporter plasmid containing −3.0-kb sequences of the mouse Tf (mTf) promoter [31] were generously provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA). The CAT reporter plasmids containing −3.0-kb mTf promoter and its deletions were constructed as follows. 1) The 3.0-kb mTf-CAT construct was made by ligating a −3.0-kb *Bam*HI-*Bam*HI fragment to the *Bam*HI site in PGL2-CAT plasmid (Promega, Madison, WI). 2) The 2-kb upstream *Hin*dIII-*Hin*dIII fragment in the 3-kb mTf promoter was ligated in the upstream *Hin*dIII site of −581-bp mTf-CAT to obtain the 2.6-kb mTf-CAT plasmid. 3) The −1.6-kb mTf-CAT plasmid was derived from the −3.0-kb mTf-CAT (no. 1 above) by digesting out the upstream −1.4-kb *Pst* I fragment.

Constructs for each of the plasmids that did not contain the specific response element of promoter fragment (i.e., promoterless plasmid) were also generated. In all cases, experiments were performed to examine the actions of various treatments on these promoterless plasmids. As discussed in *Results*, these promoterless plasmids generally did not respond to the treatments. If a small response was observed, the data are shown in the figures or stated in *Results*.

Transfection

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 [32]. Briefly, 1.5 μg reporter plasmid in 150 μl of transfection buffer (250 mM $CaCl_2$ mixed 1:1 [v:v] with double-strength Hepes [28 mM NaCl, 50 mM Hepes, and 1.47 mM Na_2HPO_4, 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 0.1% 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-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 various time intervals before harvesting for CAT assays.

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 single-strength cell lysis buffer (Promega) was added to each well, and incubation was carried out for 15 min at room temperature. The wells were then scraped and the buffer was collected in 1.5-ml microfuge tubes. Tubes were heated to 65°C for 10 min to inactivate endogenous acetylases and then spun 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), and 0.1 μCi [^{14}C]-chloramphenicol (0.1 μCi/μl; ICN, Costa Mesa, CA) and incubated overnight at 37°C. The mixture was extracted once with 300 μl xylene and back-extracted with 100 μl 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 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; Nunc, Roskilde, Denmark). The cells were treated after 48 h in culture with either FSH or dbcAMP or, for controls, were not treated. After 72 h the cells were scraped 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. [33]. Typically 70–100 μg of protein was obtained from 10^8 plated cells. The double-stranded DNA probes used in gel retardation assays were the SRE (5′-ATGTCCATATTAGGACACATCTG-3′) and the ETS-SRE element (SRE with 5′-CAGGAT sequence) of the c-*fos* promoter; CRE (5′-CAGTGACGTAGGAAGTGACGTAGGAA-3′); AP1 dimer (5′-TCGAGTTAGTCATGAGTCAC-3′); and OCT-1 (5′-AATTCCTGATTTGCATATTCATGAGAG-3′). 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 (Boehringer-Mannheim, Indianapolis, IN). The complementary oligonucleotides were annealed, electrophoretically purified, and then used as probes in gel shift assays.

The gel retardation assay used was a modification of the protocol described by Garner and Rezvin [34]. The final reaction volume of 20 μl contained 0.5 ng (approximately 50 000 cpm) of 5′ ^{32}P-labeled double-stranded probe, 100 ng sonicated salmon sperm DNA, 2 μg Poly dI-dC (U.S. Biochemicals, 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 was electrophoretically separated on a 5% polyacrylamide gel. The gel was dried and autoradiographed. For the competition experiments, excess unlabeled oligonucleotide was added in the binding reaction. The excess oligonucleotide concentration required to obtain complete displacement was found to be 100-fold molar excess for AP1, OCT-1, CRE, and ETS-SRE, while the SRE required 500-fold molar excess.

Antisense Procedure

A c-*fos* antisense oligonucleotide, 3′TACTACGGTGCA5′, was prepared and used as previously described [30, 35]. A c-*fos* sense oligonucleotide was prepared for use as a negative control. Transfected Sertoli cells were treated with either the antisense or sense oligomer (4 μM) immediately after transfection and were re-treated every 8 h for a total of 72 h before harvest of the cells for CAT assay.

Statistical Analysis

Each data point was converted to a percentage of the control value, and the mean and SEM from multiple experiments were determined as indicated in the figure legends. Data were examined by ANOVA as indicated in the figure legends.

RESULTS

To examine how FSH activates c-*fos* gene expression, CAT reporter gene constructs containing different individual regulatory elements of the c-*fos* promoter were generated, including the CRE, TRE, and SRE. A schematic representation of the location of these elements within the c-*fos* promoter is shown in Figure 1A. The sequence of the individual elements in the constructs is presented in the *Methods* section. These constructs were transiently transfected into cultured Sertoli cells. After transfection, the cells were left untreated (control) or were treated with either FSH or dbcAMP for 4 or 72 h. After a 4-h treatment (Fig. 1B), dbcAMP was found to have the most dramatic effect on the CRE-CAT, with small effects on the SRE-CAT and TRE-CAT. The whole *fos*-CAT promoter was also stimulated by dbcAMP. FSH was found to stimulate the CRE-CAT, TRE-CAT, and SRE-CAT in a similar manner (Fig. 1B). Interestingly, FSH had a more dramatic effect on the SRE-CAT than dbcAMP did. The effect of FSH on the *fos*-CAT after 4 h was greater than effects on

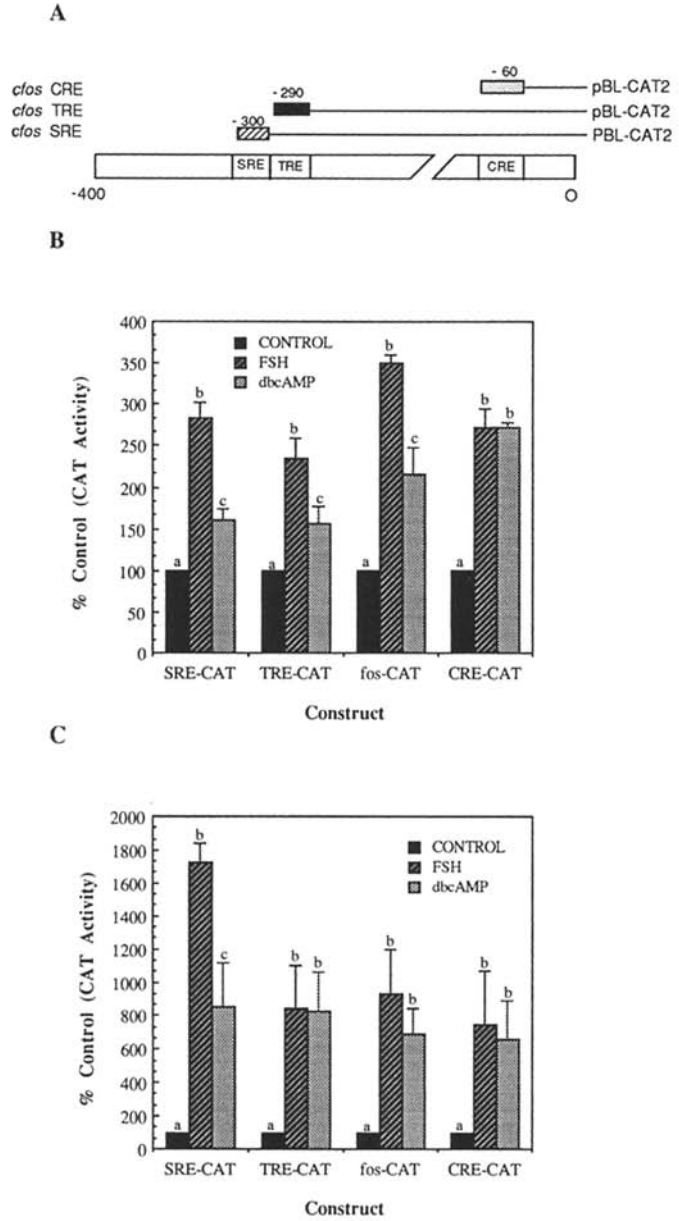

FIG. 1. A) Schematic representation of the c-*fos* promoter and the location of the CRE, SRE, and TRE in promoter-CAT constructs. The designation for the promoter constructs is listed at the left and plasmid utilized indicated at the right. Regulation of the c-*fos* promoter constructs (CRE, TRE, and SRE) in Sertoli cells cultured in the absence (control) or presence of FSH and dbcAMP for 4 h (B) and 72 h (C). Data are expressed as percentage of control and presented as the mean ± SEM from six different experiments done in replicate. The statistical analysis (ANOVA) is shown, with different letters indicating a statistical difference ($p < 0.001$) within each CAT construct group individually.

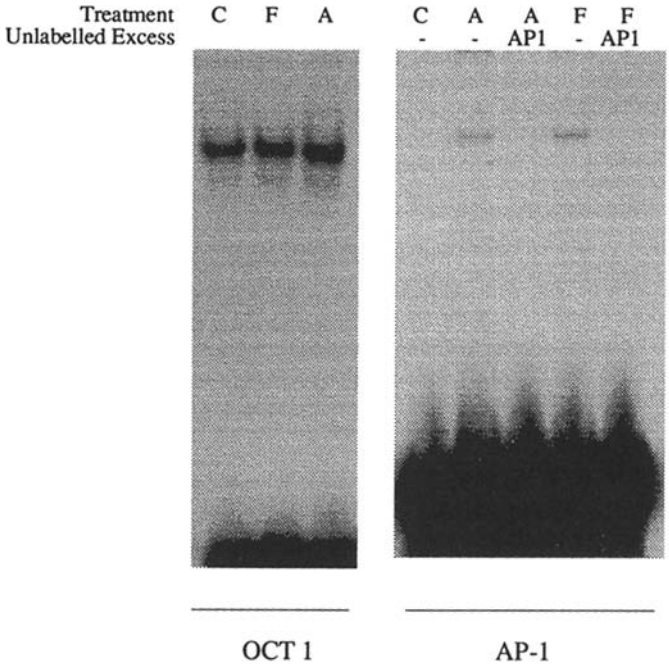

FIG. 2. Gel mobility shift assay with ^{32}P-radiolabeled Oct-1 and AP1 oligonucleotides. Nuclear extracts from Sertoli cells cultured in the absence (C) or presence of FSH (F) or dbcAMP (A) were used. The DNA/protein complexes were electrophoretically separated on 5% polyacrylamide gels and then dried and autoradiographed. The data are representative of five different experiments.

the individual elements. After a 72-h treatment (Fig. 1C), dbcAMP was found to stimulate CRE-CAT, TRE-CAT, SRE-CAT, and *fos*-CAT to the same extent. FSH stimulated CRE-CAT, TRE-CAT, and *fos*-CAT in a manner similar to dbcAMP. Interestingly, FSH had a more dramatic effect on the SRE-CAT, and this response was twice the response seen with dbcAMP (Fig. 1C). Promoterless control plasmids were not significantly affected by dbcAMP or FSH treatments (data not shown). The CAT activity under control conditions was found to be similar for SRE, CRE, and c-*fos*, while the TRE had a 2–3-fold higher basal level of activity. It is likely that the basal TRE level was higher because the TRE is responsive to the protein kinase C pathway and also has an AP-1 motif to which fos/jun can bind and activate fos expression. As expected, the activity at 72 h was significantly higher than at 4 h. Therefore, FSH can act at response elements other than the CRE, in particular the SRE, to regulate c-*fos* promoter activation.

To extend these observations, gel retardation/mobility shift assays were performed. Nuclear extracts were obtained from Sertoli cells cultured in the absence (control; C) or presence of FSH or dbcAMP. A gel shift with an oligonucleotide to Oct-1 was used to check the quality of the nuclear extract (Fig. 2). All the extracts resulted in a similar amount of Oct-1 gel shift. To confirm that c-*fos* was induced, a gel shift with an AP1 oligonucleotide was performed, since the c-*fos/jun* complex binds to the AP1 site. Incubation of the labeled AP1-DNA fragment with nuclear extracts of Sertoli cells treated with FSH or dbcAMP resulted in the formation of a DNA/protein complex (i.e., gel shift). The observed gel shift was specific for AP1 since it could be effectively inhibited by unlabeled AP1 (Fig. 2). The optimum concentration for unlabeled oligonucleotide competition experiments is specified in *Materials and Methods*. The presence of c-

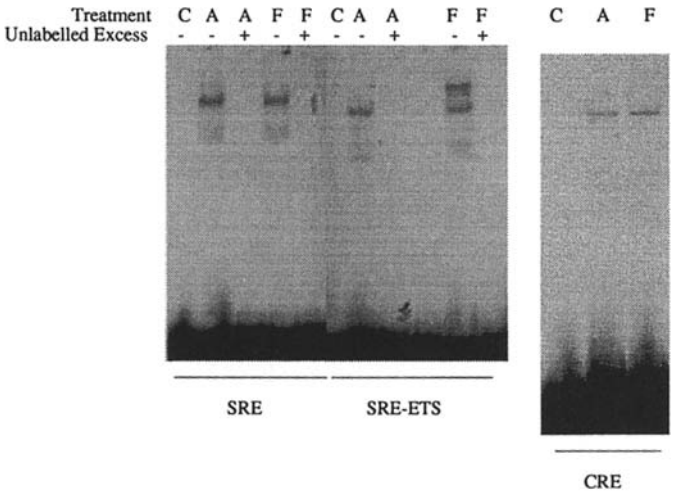

FIG. 3. Gel mobility shift assay with ^{32}P-radiolabeled SRE, ETS-SRE, and CRE oligonucleotides. Nuclear extracts from Sertoli cells cultured in the absence (C) or presence of FSH (F) or dbcAMP (A) were used. The DNA/protein complexes were electrophoretically separated on 5% polyacrylamide gels and then dried and autoradiographed. The data represent a minimum of three different experiments.

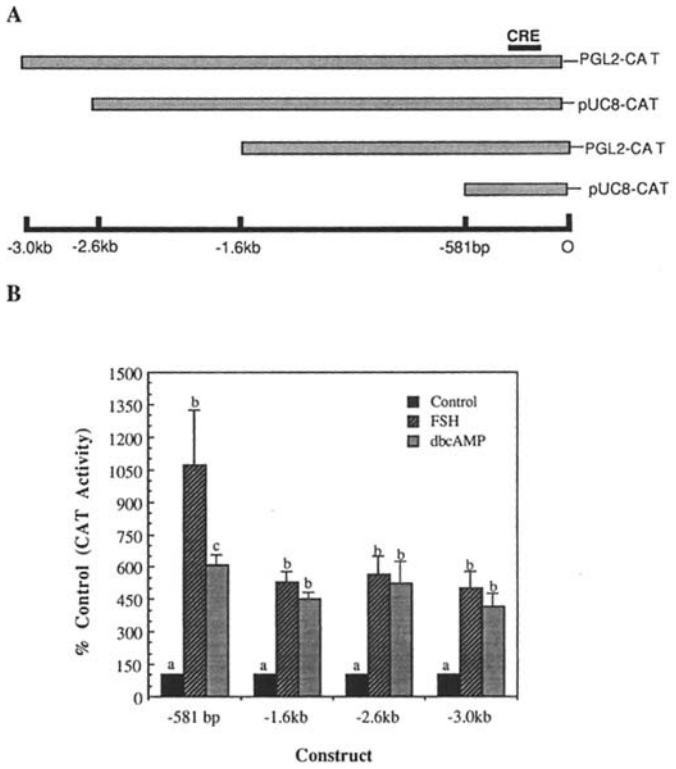

FIG. 4. A) Schematic representation of the transferrin promoter-CAT constructs. B) Regulation of the various transferrin promoter-CAT constructs in Sertoli cells cultured in the absence (Control) or presence of FSH or dbcAMP. Data are expressed as percentage of control (nontreated) cells and presented as the mean ± SEM from five different experiments done in replicate. The statistical analysis (ANOVA) is shown with different letters indicating a statistical difference ($p < 0.001$) within each CAT construct group individually.

fos in this AP1 gel shift was confirmed with an immunoblot of the gel shift with a c-*fos* antibody (data not shown). This confirmed the induction of c-*fos* by both FSH and cAMP. The activation of the SRE of the c-*fos* promoter was also investigated with a gel mobility shift assay using an SRE oligonucleotide (Fig. 3). FSH and dbcAMP induced a gel shift that was inhibited with excess unlabeled SRE. To determine whether a ternary complex could be induced, an SRE-ETS oligonucleotide gel shift was done. The ETS domain, previously shown to bind Ets oncogenes, is a 5′-CAGGAT sequence to the SRE and is required to obtain a ternary complex with the serum response factor (SRF) binding to the SRE. FSH promoted an SRE-ETS gel shift that was distinct from that of dbcAMP (Fig. 3). Therefore, FSH promotes the formation of an apparent ternary complex with an SRE-ETS sequence that is distinct from dbcAMP actions (Fig. 3). FSH and cAMP were also found to induce a gel shift with a CRE oligonucleotide (Fig. 3) that was inhibited with excess unlabeled CRE (data not shown). Therefore, FSH induced protein binding to the SRE and CRE oligonucleotides. The results correlate with the reporter gene data outlined above and suggest that FSH may act through both the SRE and CRE to promote c-*fos* gene expression.

In order to determine the location of response elements within the transferrin promoter that are activated by treatment with FSH, four CAT constructs containing either a 581-bp, 1.6-kb, 2.6-kb, or 3-kb upstream region of the mouse transferrin promoter were used (Fig. 4A). The minimal 581-bp promoter was previously shown to contain a CRE [26]. Cyclic AMP was found to stimulate all the transferrin promoter constructs in a similar manner (Fig. 4B). FSH had the most dramatic effect on the minimal 581-bp promoter, and this level was nearly twice that obtained with dbcAMP. FSH actions on the 1.6-, 2.6-, and 3-kb promoter constructs were the same as those found with cAMP. Therefore, the minimal 581-bp promoter appears to contain the required elements for cAMP and FSH to activate transferrin gene expression. The basal CAT activity under control conditions for all the constructs was found to be similar. A promoter construct that had the 581-bp minimal promoter deleted and contained only a 1-kb promoter fragment between −1.6 kb and −2.6 kb was found not to be activated by either FSH or dbcAMP treatment (data not shown). FSH appears to primarily utilize the 581-bp minimal promoter to activate transferrin expression and has a more dramatic effect than cAMP alone.

To confirm that c-*fos* is involved at a molecular level in FSH stimulation of transferrin, a CAT reporter gene construct containing −2.6 kb of the transferrin gene promoter was transiently transfected into cultured Sertoli cells. After transfection, the cells were left untreated (control) or were treated with FSH, a combination of FSH and the antisense c-*fos* oligonucleotide, or a combination of FSH and the sense c-*fos* oligonucleotide for 72 h before analysis of the cell extracts for CAT activity. FSH and the combination of

FSH and the sense oligonucleotide activated the transferrin CAT construct (Fig. 5). The c-*fos* antisense oligonucleotide partially inhibited (approximately 50% inhibition) the actions of FSH (Fig. 5). Therefore, c-*fos* appears to be partially involved in the ability of FSH to activate the transferrin promoter, but other more direct *cis*-acting elements (e.g., CRE) will also be involved.

DISCUSSION

FSH is known to have the ability to influence Sertoli cell differentiation through a classical cAMP-dependent signal transduction pathway [1]. However, FSH can also influence other signal transduction pathways (e.g., calcium mobilization) that can alter Sertoli cell function [1, 10–16]. The current study investigated the ability of FSH to influence the transcriptional regulation of Sertoli cell differentiation. The possibility that FSH may also influence multiple transcriptional events was considered using both an immediate-early response gene and a downstream gene. FSH is known to alter early-event genes [17], in particular, c-*fos* [18–20]. FSH causes a transient increase in c-*fos* mRNA in Sertoli cells [19]. A downstream differentiated function of Sertoli cells is transferrin gene expression that has also been shown to be stimulated by FSH [8, 23, 24]. The actions of FSH on the promoters of c-*fos* and transferrin were examined to provide insight into the transcriptional regulation of Sertoli differentiation.

Analysis of the actions of FSH on various individual response elements of the c-*fos* promoter indicated that the CRE-CAT, TRE-CAT, and SRE-CAT can all be activated as well as the whole c-*fos* promoter. The CRE was activated to the same extent by FSH and cAMP. This confirms previous observations on the ability of a CRE to mediate the actions of FSH on Sertoli cell genes [8, 9]. FSH and dbcAMP also promoted a gel shift with a CRE oligonucleotide. CRE-binding proteins have been postulated previously to be involved in the actions of FSH [36]. The CRE on some promoters have been shown to bind the CRE-binding protein (CREB). Whether the CREB or other proteins participate in the CRE gel shift shown in the current study remains to be elucidated. Therefore, as expected, the CRE of the c-*fos* promoter may have an important role in activation of c-*fos* expression. In addition to the CRE, the TPA response element TRE was also activated by FSH. The response to FSH at 4 h was greater than that to cAMP but was similar at 72 h. The TRE utilized was located at −290 bp of the c-*fos* promoter and is also known to contain an AP1 site [37]. Therefore, this element can respond to an autoregulatory c-*fos* expression. Results imply that FSH can influence this TRE element to influence c-*fos* expression. Whether this activation entails a calcium mobilization and/or protein kinase C activation vs. an autoregulation involving c-*fos*/*jun* remains to be elucidated.

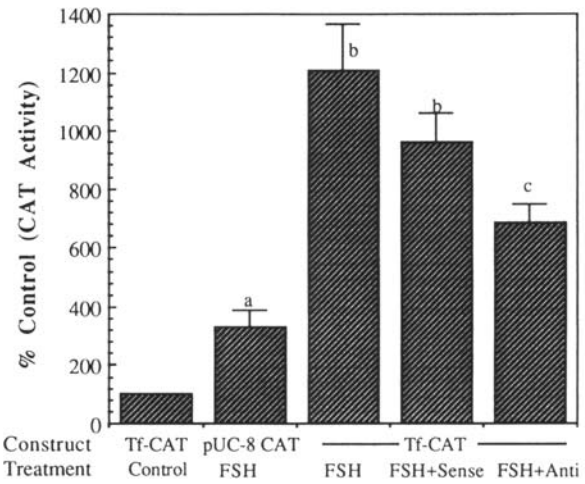

FIG. 5. Regulation of the 2.6-kb transferrin promoter-CAT construct (Tf-CAT) in Sertoli cells cultured in the absence (Control) or presence of FSH (FSH), FSH and c-*fos* antisense oligonucleotide (FSH + Anti), or FSH and c-*fos* sense oligonucleotide (FSH + Sense). The effect of FSH on the promoterless plasmid (pUC-8 CAT) is indicated. Data are expressed as percentage of control and as ANOVA results with different letters indicating a statistical difference ($p < 0.001$) between treatments. Data are presented as the mean ± SEM from three different experiments done in replicate.

The SRE was also activated by FSH and was the most active promoter at 72 h. This increased activity may have been due to a differentiation response at 72 h, whereas it is likely that at 4 h the activation was exclusively an immediate signal transduction response. FSH had a more dramatic effect than cAMP at both 4- and 72-h treatments. FSH also promoted a gel shift with an SRE oligonucleotide. This observation supports the reporter gene results and demonstrates the ability of FSH to promote the presence of transcriptional regulators that bind the SRE. SRE is a 29-bp region within the c-*fos* promoter. The 14-bp inner core contains the sequence [$CC(A/T)_6GG$], which is referred to as the CArG box. Transcriptional regulators bind to this sequence, and their binding is stabilized by the two outer arms of the element, which are palindromic [38]. A 67-kDa protein has been isolated that binds to the SRE—the serum response factor (SRF) [39]. SRF is a highly conserved, dimeric protein with novel DNA-binding structure. The DNA-binding domain of SRF, amino acids 93–222, is also the region necessary for dimerization and interaction with other proteins to form ternary complexes [39]. Because SRF is ubiquitously expressed, cell-specific activation of the c-*fos* SRE by SRF appears to be mediated by these other protein factors. The binding of the ternary complex to the SRE requires the presence of adjoining 5′-flanking sequence of the SRE-binding site, the ETS domain-binding motif. The protein kinase C-dependent and -independent pathways target different parts of this SRE-ETS ternary complex [40]. One protein required for serum-induced activation of the SRE by SRF is the 62-kDa, p62 ternary complex factor ($p62^{TCF}$). Gel shift data demonstrated a 50-fold increase in the affinity of SRE

for the SRF/p62^{TCF} complex over that for SRF alone [39]. Another protein that binds SRF is *elk*-1 protein, which may be structurally related to p62^{TCF} [41, 42]. The *elk*-1 protein is phosphorylated through the activation of MAP kinase pathway by growth factors. The TCF formed between phosphorylated *elk*-1 and SRF results in a readily distinguishable DNA-protein complex as compared to SRF alone [43]. The identity of the FSH-induced SRE-binding protein as SRF or other binding proteins remains to be elucidated. FSH also induced a unique gel shift with the SRE-ETS that was not observed in control nontreated cells or in cAMP analog-treated cells. Therefore, FSH appears to promote the formation of a protein complex (i.e., ternary complex) distinct from cAMP that activates the SRE within the c-*fos* promoter. The formation of these ternary complexes may enable a common regulatory element, such as the SRE, to have cell-specific activity using ubiquitously expressed DNA-binding protein. Alternatively, different or unique binding factors may be another method by which a common regulatory element may differentially activate cell-specific gene expression.

Combined observations indicate that FSH can activate c-*fos* expression through multiple response elements (e.g., CRE and SRE). Previously, multiple sequence elements in the c-*fos* promoter have been shown to be induced by cAMP [44]. In addition, the SRE was found not to mediate cAMP induction of c-*fos* [45]. These observations support the current results indicating that FSH appears to act at multiple response elements and transcriptional activation pathways. The sequences of the c-*fos* response elements used are presented above; no known response elements are present within these sequences other than those listed. The possible presence of unknown response elements cannot be ruled out. Mutagenesis experiments with these elements are needed to unequivocally identify a specific element as mediating FSH actions. However, observations do indicate that multiple elements appear to mediate FSH actions, and this correlates with the ability of FSH to influence multiple signal transduction pathways.

The downstream effects of FSH were investigated using the transferrin gene as a differentiated marker of Sertoli cells. The regions controlling liver-specific transcription of the human transferrin gene are composed of multiple positive- and negative-acting elements, mostly interacting with DNA-binding proteins present in either human or rat liver nuclear extracts [46, 47]. The regulation of the transferrin gene expression in unstimulated Sertoli cells has been shown to be distinct from that for liver gene expression [48]. Although the first 581 bp of human transferrin promoter (hTf) and mouse transferrin promoter (mTf) have little similarity, they both contain a CRE site that was found in the current study to be responsive to FSH. The results for the CAT-mTF deletion mutants suggest that the enhancer activity for FSH and cAMP is located in the 581-bp minimal promoter. The upstream 2.5 kb of promoter was found to provide equivalent or decreased activity. Therefore, the 581-bp minimal promoter appears to be sufficient for FSH-regulated transferrin expression. One mechanism FSH can use to regulate transferrin expression is through the CRE located in the minimal promoter. What function c-*fos* expression may have in mediating FSH actions was investigated with an antisense c-*fos* oligonucleotide. The antisense c-*fos* was found to partially (50%) inhibit FSH-induced activation of the transferrin promoter in Sertoli cells. The antisense c-*fos* procedure does have the capacity to completely inhibit c-*fos* actions as shown with another Sertoli cell regulator (i.e., PModS) [49] (data not shown). The ability of the antisense c-*fos* to partially inhibit FSH actions suggests that c-*fos* in part mediates FSH activation of the transferrin promoter. Therefore, in addition to actions on the CRE, FSH also appears to utilize the early-event gene c-*fos*. The CRE as well as the c-*fos* response appears to be an early-event response to FSH and not a secondary indirect response to one or the other. FSH appears to regulate multiple transcriptional activation pathways to modulate Sertoli cell differentiation.

In considering the transcriptional regulation of Sertoli cell differentiation, the ability of FSH to influence multiple signal transduction and transcriptional activation pathways is an issue to address. These multiple pathways may act in a compensatory manner to assure the activation of Sertoli cell differentiation by FSH. This may have evolved because of the importance of Sertoli cell differentiation and spermatogenesis to male reproduction. Alternatively, these transduction pathways may act synergistically to amplify FSH actions on Sertoli differentiation. In support of this, the 581-bp transferrin minimal promoter was activated to a greater extent by FSH than by cAMP. In addition, the c-*fos* antisense experiment only partially inhibited FSH actions. This suggests that possible synergistic actions of multiple transcriptional activation pathways are possible.

The ability of FSH to activate multiple transduction pathways implies that FSH may be a general activator of the Sertoli cell. This may be essential in the progression and maintenance of Sertoli differentiation. Other regulatory agents with more specific transduction pathways may be needed for the initial induction of Sertoli cell differentiation. The actions of FSH may also be needed to make the Sertoli cell responsive to other regulatory agents. Further analysis of the transcriptional regulation of Sertoli cell differentiation will involve the identification of important transcription factors and *cis*-acting elements in a variety of Sertoli cell differentiated genes.

ACKNOWLEDGMENTS

We thank Lisa Halburnt and Betty Chu for technical assistance and Ellisa Sagle, Pamela Garay-Kobuchi, and Karen Johnson for assistance in the preparation of the manuscript. We acknowledge the assistance of the UCSF Reproductive Endocrinology Center (HD-11979).

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