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Role of Basic Helix-Loop-Helix (bHLH) and CREB Transcription Factors in the Regulation of Sertoli Cell Androgen-Binding Protein Expression

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ABSTRACT Differentiation of Sertoli cells is marked by the presence of novel gene products such as transferrin and androgen-binding protein (ABP). Transcriptional regulation of Sertoli cell differentiation is, in part, controlled through the binding of specific transcription factors to response elements within these genes promoters. Transferrin gene expression has been shown to be regulated by the binding and interactions of basic helix-loop-helix (bHLH) and cAMP response element binding protein (CREB) to an E-box and cyclic AMP response element (CRE), respectively. Interaction between the bHLH and CREB is facilitated through subsequent binding of CREB-binding protein (CBP)/p300. The hypothesis tested in the current study is that ABP expression is regulated by a similar mechanism. The ABP promoter activation was analyzed through the use of transfection assays, site-directed mutagenesis, and electromobility shift assays (EMSA). Transient transfections of rat Sertoli cells used a reporter construct containing the proximal 619 bp of the ABP promoter. Observations suggest that cAMP and follicle stimulating hormone (FSH) upregulate the expression of ABP. Mutational studies of the three E-boxes and the CRE of the 619-bp ABP promoter indicate that all of these elements are critical for stimulation of promoter activity. EMSA revealed a weak interaction between an E-box-2 and the CRE that are overlapping in the promoter. An artificial promoter that contains only an E-box and CRE was created to further test this hypothesis. The artificial promoter was stimulated by both FSH and cAMP. Experiments with mutants of the artificial promoter demonstrate that both response elements contribute to the optimal activation of the promoter construct. The overexpression of the bHLH inhibitor Id (i.e., inhibitor of differentiation) that binds bHLH proteins and eliminates DNA binding was found to suppress hormone activation of the ABP promoter. Combined observations of the ABP promoter and artificial promoter provide insight into a common mechanism for gene regulation in differentiated Sertoli cells involving a role for both the bHLH and CREB family of transcription factors. *Mol. Reprod. Dev. 68: 269–278, 2004.*

Key Words: testis; Sertoli; ABP; FSH; bHLH; CREB; CRE; E-box; transcriptional regulation

INTRODUCTION

Sertoli cells help form the seminiferous tubules and provide the structural and nutritional support necessary for the development of germ cells (i.e., spermatogenesis). Sertoli cells differentiate in response to follicle stimulating hormone (FSH), testosterone, and locally produced paracrine factors (Skinner, 1991). The Sertoli cells morphologically change during (Skinner, 1991) puberty into elongated polarized epithelial cells extending from the basal lamina to the tubular lumen. The induction of Sertoli cell differentiation at the onset of puberty promotes a post-mitotic terminally differentiated cell that is correlated with the expression of cell-specific genes. Sertoli cells secrete a large number of proteins that include transport-binding proteins such as transferrin and androgen-binding protein (ABP), proteases and growth factors to help maintain and regulate the microenvironment within the seminiferous tubule (Skinner, 1991). The transcriptional regulation of these Sertoli cell gene products is essential for Sertoli cell differentiation and maintenance of testis functions.

Transcription factors bind and modulate specific response elements on promoters to regulate the expression of specific genes. One important transcription factor family involved in cellular differentiation is the helix-loop-helix (HLH) family. Transcription factors in this family consist of two alpha helices connected by a loop. When a basic region is present, they are referred to as basic HLH (bHLH) transcription factors. bHLH factors bind DNA following dimerization (homodimers or heterodimers) and activate transcription. The bHLH dimer recognizes a specific response element termed the E-box. The E-box has the consensus hexanucleotide sequence CANNTG. There are two classes of bHLH transcription factors. Class A consists of ubiquitously expressed proteins including HEB (Hu et al., 1992) and the products of the E2A gene, E12 and E47 (Murre et al., 1989). Class B bHLH proteins are tissue specific and developmentally regulated. Examples include MyoD

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(Olson and Klein, 1994), NeuroD (Lee, 1997), and Hxt (Cross et al., 1995). Class B bHLH proteins generally do not efficiently form homodimers, so it is necessary to form heterodimers with the class A bHLH proteins to regulate transcription (Chakraborty et al., 1991).

The HLH proteins lacking the basic region do not generally bind DNA. These HLH proteins can dimerize with a variety of bHLH proteins. An HLH-bHLH dimer cannot bind to DNA. One such HLH class of proteins is the inhibitor of differentiation (Id) family. Id proteins are mainly expressed during cell proliferation and help promote the cell cycle. Sertoli cells that are terminally differentiated have negligible Id expression (Chaudhary et al., 2001). Therefore, it is possible that the bHLH factors present in the cells are available to promote transcription of genes in post-mitotic differentiated Sertoli cells.

A common transcription factor known to regulate Sertoli cells is the cyclic AMP response element-binding protein (CREB). CREB has a basic leucine zipper motif that binds as a dimer to the cyclic AMP-response element (CRE) (Berruti, 1998). CRE contains a consensus sequence of TGACGTCA, but variants of this sequence are able to allow binding of CREB (Daniel et al., 1998). CREB is primarily activated through a cAMP signaling pathway involving protein kinase A. For example, FSH binds to the FSH receptor (FSHR) to stimulate a signaling pathway that activates the cAMP cascade that leads to phosphorylation of nuclear transcription factors, including CREB (Berruti, 1998). Phosphorylated CREB binds to the CRE and promotes transcription. CREB-binding protein (CBP) with histone acetyl transferase (HAT) activity and p300 are transcriptional co-activators that can bind to CREB to further enhance transcription. CBP and p300 are also co-activators of other transcription factors such as basic HLH proteins (Daniel et al., 1998). An interaction between bHLH, CREB, and CBP/p300 proteins is proposed in the testicular transferrin promoter model (Chaudhary and Skinner, 2001).

The iron-binding and transport protein transferrin is expressed in both hepatocytes and Sertoli cells (Skinner, 1991). The mechanism by which this gene is activated differs between the two cell types. The transferrin gene in hepatocytes is regulated by the binding of iron (Idzerda et al., 1986). Transferrin regulation in Sertoli cells is controlled by hormones such as FSH (Chaudhary et al., 1997) and is responsive to cAMP (Chaudhary and Skinner, 1999). The transferrin promoter contains the consensus sequence for classic TATAA and CCAAT sequences (Guillou et al., 1991). It has been shown that expression of the transferrin gene can be attributed to the presence of an E-box and a CREB binding CRE-like proximal region II (PRII) (Chaudhary and Skinner, 1999). Mutational studies have shown that the presence of both the E-box and PRII are required for optimal expression of the transferrin promoter. Furthermore, it was shown that CBP/p300 appears to be required for the interactions between bHLH and CREB proteins (Chaudhary and Skinner, 2001). The E-box and PRII of the transferrin promoter are located 220 base pairs (bp) apart. This separation is ideal for DNA bending around a nucleosome and allows close contact between both elements. Since the ABP promoter also contains E-box and CRE response elements, it is proposed that a similar mechanism of action may be used to regulate ABP expression.

The ABP is a marker of Sertoli cell differentiation (Gunsalus et al., 1981). ABP functions in the transport of testosterone through the seminiferous tubule lumen into the epididymis (Joseph, 1997). This same gene encodes the sex hormone-binding globulin (SHBG) that is found in the liver and aids in the transport of steroids through plasma (Hammond et al., 1989). Previous studies have demonstrated that the ABP promoter is 1,486 bp (Joseph et al., 1988) and that the distal 867 bp of the ABP promoter inhibits transcription (Fenstermacher and Joseph, 1997). The proximal 619 bp of the ABP promoter is active in Sertoli cells, but has not been characterized in regards to the identification of proximal response elements. The current study is the first to investigate the role of individual response elements in the ABP promoter. The role of a TATAA box in the proximal promoter has been investigated and it was determined that the presence or absence of a TATAA box does not influence the transcription of ABP (Fenstermacher and Joseph, 1997, 1998). Furthermore, the ABP promoter has been shown to be sensitive to cAMP suggesting a CRE is likely present (Joseph et al., 1988). The ABP promoter has been previously examined by deletion mapping (Fenstermacher and Joseph, 1997; Tung et al., 1984). While deletion mapping is useful to determine regions within the promoter that likely contain response elements, it is essential to examine specific putative response elements to fully understand the promoter regulation.

The hypothesis tested is that optimal ABP promoter activity requires both the bHLH factors that are bound to one of the E-boxes and the CREB factor bound to the CRE. This model is similar to that proposed for the transferrin promoter (Chaudhary and Skinner, 1999, 2001), except that the distance separating the E-box and PRII in the transferrin promoter is different from that in the ABP promoter. Additionally, the ABP promoter model is complicated by the potential interactions between one of the E-boxes and the CRE that overlap. The current study provides additional insight into a common mechanism for Sertoli cell-specific gene promoter activation through interactions between the bHLH and CREB transcription factors using an artificial promoter construct.

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 (Dorrington et al., 1975; Tung et al., 1984) with some modifications. Any procedures involving the use of animals were reviewed and approved by the WSU Animal

Care Committee. The isolated Sertoli cells were greater than 98% pure and plated in Ham F-12 without serum. Cells were maintained in a 5% CO_2 atmosphere in Ham F-12 medium at 32°C (Invitrogen, Carlsbad, CA). Sertoli cells were treated in Ham F-12 + 0.01% BSA with either FSH (50 ng/ml; o-FSH-16, National Pituitary Agency, Torrance, CA), dibutyryl cAMP (Sigma, St. Louis, MO) (db-cAMP; 0.5 mM), 10% bovine calf serum (serum) (Hyclone), or left in media alone (Ham F-12, control). Sertoli cells were cultured for a maximum of 5 days, with a media change and treatment after 48 hr of culture. Cell number, purity (i.e., >98%), and viability remained constant during the culture in the absence or presence of treatment (Skinner and Fritz, 1985; Skinner et al., 1988) (data not shown).

Reporter Gene Construct

The pBluescript plasmid containing the ABP promoter (1.5 kb) was generously provided by Dr. David Joseph. The entire ABP promoter was transferred to the pGL3 basic plasmid (Promega, Madison, WI), containing the luciferase reporter gene. The truncated ABP promoter with a length of 619 bp was generated by engineering new restriction enzyme sites. The mutations of the CRE and E-boxes in the ABP promoter were performed using a Quik-Change Site-Directed Mutagenesis kit (Stratagene, San Diego, CA). Site-specific mutations were confirmed by sequencing.

Artificial Reporter Construct

An artificial promoter with a 100-bp sequence was designed, which contained only the transferrin E-box and a consensus CRE. The oligos were as follows: 5′-TAGGTAAG CTTTCACGGCTAAGCAGCTGTACCATGCTGCTGCAGGGTACCCCCGGGGATGCGGGGTTGACGTCACAAATGGACTACGCAACTCGAGTAGG and 3′-CCTACTCG AGTTGCGTAGTCCATTTGTGACGTCAACCCCGCATCCCCGGGGGTACCCTGCAGCAGCATGGTACAGCTGCTTAGCCGTGAAAGCTTACCTA. Two core sequences were designed to be inserted between the E-box and CRE response elements. The 46-bp core is as follows: 5′-TAGGTGGTACCACCATAGAAGAAGGATTATTACCTGGTAC CTAGGT and 3′-ACCTAGGTACCAGGTAATAATCCTTCTTCTATGGTGGTACC ACCTA. The 90-bp core is as follows: 5′-TAGGTGGTACCACCATAGAAGAAGGA TTATTCTACCATAGAAGAAGGATTATTCTACCATAGAAGAAGGATTATTACCTGGTACCTAGGT and 3′-ACCTAGGTACCAGGTAATAATCCTTCTTCTATGGTAG AATAATCCTTCTTCTATGGTAGAATAATCCTTCTTCTATG - GTGGTACCACCTA. The artificial promoter was first inserted into pGEM-T EZ (Promega). Next, directional cloning was used to insert the artificial promoter into pLuc-MCS (Stratagene, San Diego, CA), which has a TATA box and expresses luciferase. Mutations of the artificial promoter were done by digesting one response element and using S1 nuclease (Invitrogen) to create a blunt end for ligation. Constructs were confirmed by sequencing. The absence of any additional response elements was determined with a Blast search using known response element consensus sequences.

Transfection

Sertoli cells cultured in 24-well plates at a density of 10^6 cells/well for 48 hr were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described (Chaudhary and Skinner, 1999). Briefly, 0.75 μg of reporter plasmid in 150 μl 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 medium and then incubated at 32°C for 3–5 hr. Following incubation, the cells were subjected to hyperosmotic shock. The medium was aspirated, and 1 ml of 10% glycerol in Hanks' Balanced Salt Solution (HBSS) (Invitrogen) was added for 3 min. Wells were washed twice in HBSS before fresh Ham's F-12 + 0.01 BSA was added. Treatments included FSH (50 ng/ml; o-FSH-16, National Pituitary Agency, Torrance, CA), dibutryl cAMP (db-cAMP; 500 mM), or 10% serum. Untreated cells served as a negative control. The cells were incubated for 24, 48, or 72 hr or 5 days before harvesting for luciferase assays (Promega). Co-transfection of the ABP promoter construct and an Id construct with a constitutive promoter was performed as outlined above. Transfection efficiency was measured using a Renilla-Luciferase plasmid (Promega) and comparing DNA amounts by SYBR green (Molecular Probes, Eugene, OR) detection. Luciferase assays were performed to quantitate levels of the reporter vector induction. Following an 8-, 24-, 48-, or 72-hr, or 5-day incubation, the Sertoli cells were harvested for luciferase assays using the Promega Luciferase kit. Twenty microliters of the primary Sertoli cell lysate were tested with 100 μl of luciferase substrate. The Wallac 1420 was used to measure the level of luciferase activity.

Transfection variability was tested using a Renilla-luciferase construct. Data combined from fourteen assays demonstrated a coefficient of variation of 13% for the transfection efficiency. Data combined from eight DNA assays demonstrated a coefficient of variation of 5.9% for DNA quantity. These two experiments and the low standard error achieved in the ABP promoter experiments suggest that the transfection efficiency was relatively constant between experiments.

Nuclear Extract Preparation

Sertoli cells were cultured in 150-mm plates and treated with FSH, db-cAMP, and 10% serum as described above. After 72 hr of treatment, the cells were washed twice with HBSS and treated and collected according to the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL) supplemented with mini-protein protease inhibitor cocktail (Boehringer, Indianapolis, IN). The protein concentration in the supernatants was calculated using the Bradford assay (Bio-Rad, Hercules, CA). Approximately 50—150 μg of total protein was

prepared for electromobility shift assays (EMSA) and immunoblots. The quality of the nuclear extracts was monitored with Oct-1 oligonucleotides.

EMSA and Immunoblotting

EMSA were performed as previously described (Chaudhary and Skinner, 1999) with nuclear extracts of isolated Sertoli cells. Sertoli cells nuclear extracts were isolated as described above. The double-stranded DNA probes with flanking sequence were designed appropriately to study regulatory sequences (E-box and CRE regions). The final reaction volume of 20 μl contained 0.5 ng (approximately 50,000–100,000 cpm) of 5′-^{32}P-labeled double stranded probe, 100 ng sonicated salmon sperm DNA, 2 μg Poly dI-dC (USB), 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 DTT, and 5 μg Sertoli cell nuclear proteins. The 20-μl binding reaction was electrophoretically separated on a 5% polyacrylaminde gel in 0.5× TBE. The gel was dried under vacuum and exposed to Kodak X-OMAT Blue film (Eastman Kodak Co., Rochester, NY) at −80°C for approximately 7–14 days. For the competition experiments, excess unlabeled oligonucleotide was added in the binding reaction. Immunoblot analysis of the EMSA was performed using unlabeled oligonucleotides in parallel with radiolabeled oligonucleotides. The protein was subsequently transferred onto PVDF membrane (Millipore, Beverly, MA). Rabbit polyclonal antibodies to CREB (Cell Signaling Technology, Beverly, MA) and E47 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at a 1:1,000 dilution. An alkaline phosphatase chemiluminescent detection kit (Pierce) was used to detect reactive bands.

RESULTS

The hypothesis tested is that both the bHLH and CREB factors are required in the transcriptional regulation of ABP expression. The proximal 619 bp of the ABP promoter contains three putative E-boxes and a CRE (Fig. 1A). The E-box most proximal to the start site of the gene is labeled E-box 1, the most distal is E-box 3. The E-box 2 and the CRE overlap at the 5′-end of the CRE. The importance of each response element was determined using transient transfections, site-specific mutagenesis of response elements, and EMSA.

Preliminary experiments were performed to determine optimal conditions for transfections. Dose-response curves for FSH and dibutyryl-cAMP (db-cAMP), as well as DNA concentrations were generated (data not shown) (Skinner et al., 1988). It was found that 50 ng/ml FSH, 0.5 mM db-cAMP, and 0.75 μg of DNA per well, along with the standard 10% serum, were optimal for the ABP promoter activation. The activity of the full-length 1.5-kb ABP promoter was compared to a truncated 619-bp proximal promoter (Fig. 2). The results indicate that at a 24-hr treatment time, the 619-bp ABP promoter basal activity was increased approximately twofold compared to the full-length promoter. These data support the previous observation that an apparent repressor exists on the full-length 1.5-kb ABP promoter (Hall et al., 1990). The truncated 619-bp promoter can be induced slightly upon the addition of FSH and serum, and induced more than fourfold by the addition of db-cAMP (Fig. 2). These data are consistent with previously published results indicating FSH does not directly induce the full-length ABP promoter (Hall et al., 1990) and only slightly increases the levels of secreted ABP in primary Sertoli cell cultures (Fenstermacher and Joseph, 1997).

The 619-bp ABP promoter activity was measured at 8, 24, 48, 72 hr, and 5 days to determine the treatment duration for optimal promoter induction (Fig. 3). The db-cAMP response increased at 8 hr and peaked at 24 hr. The db-cAMP response decreased from 24 to 72 hr and increased again at 5 days. This trend indicates both a direct and delayed (i.e., indirect) effect of cAMP on the ABP promoter. The response to FSH remained relatively identical to baseline values, except for a two-fold increase at 24 hr. The serum response remained at baseline levels through 48 hr and increased at 72 hr and peaked at 5 days where a fivefold increase in activity was observed.

Oligonucleotide sequences shown in Figure 1 were used to generate the mutations of E-box 1, E-box 2, E-box 3, and CRE. The E-box 1 and E-box 3 mutations resulted in complete inhibition of the ABP promoter (Fig. 4). These data suggest that E-box 1 and E-box 3 are necessary for optimal activity of the 619-bp ABP promoter. The baseline controls for each construct are presented in Figure 4. The mutation in E-box 1 and E-box 3 resulted in the same relative light units as control for all hormone and serum treatments. Therefore, both E-box 1 and E-box 3 independently are essential for the activity of the ABP promoter (Fig. 4). This is in contrast to the E-box2/CRE element that overlapped and did have activity above basal controls.

E-box 2 and CRE mutations decreased basal promoter activity, and FSH and cAMP stimulated a similar induction of the promoter as the wild-type (WT) promoter (Fig. 4). One possible explanation for this observation is the overlapping nature of these elements. Two base pairs of the 3′-end of E-box 2 lie within the 5′-portion of the CRE (Fig. 1). The overlapping nature of these elements could allow for cooperate binding of both classes of transcription factors, or competitive binding of one or the other factor, or a unique complex of proteins. Since the mutation in either element did not abolish activity (Fig. 4), and the nature of this element may allow for interactions, further analysis of the E-box2/CRE element was initiated with EMSA.

Sertoli cell nuclear extracts from untreated control cells or cells treated with FSH, db-cAMP, or serum were incubated with radiolabeled oligonucleotides corresponding to the E-box 2/CRE (−108 to −133) region of the 619-bp WT ABP promoter, mutated E-box 2, and mutated CRE (CRm). The 619-bp ABP promoter E-box 2 and CRE response element sequence was selected to elucidate the binding in this region due to the overlapping nature of the elements and activity of mutated

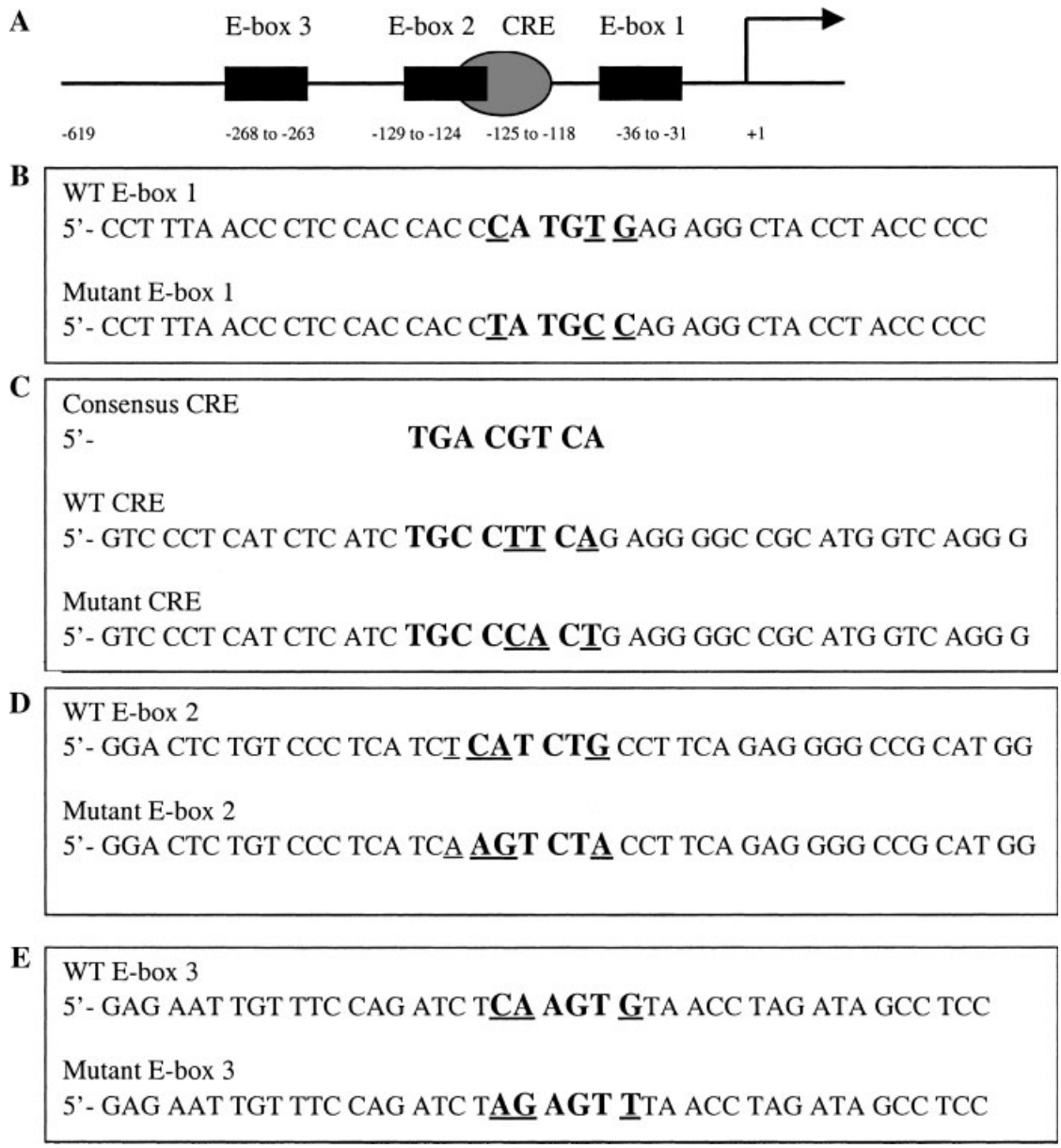

Fig. 1. A: Schematic diagram of potential regulatory sites in the proximal 619 bp of the 1.5 kb ABP promoter. The E-boxes are potential sites of bHLH binding and CRE that potentially binds CREB. The E-box 2 and the CRE overlap by two base pairs. **B–E**: Sequence of the wild-type (WT) and mutant E-boxes and CRE oligonucleotides used in electromobility shift assays and of the mutant ABP promoter constructs. Larger and bold text indicates the response element and underlined letters indicate those mutated. In (C), the consensus CRE is included for comparison. In (D), the underlined base pair was mutated outside the response element to prevent a new E-box sequence from being formed.

constructs. Radiolabeled oligonucleotides of WT ABP E-box2/CRE and mutated E-box 2 were used in an EMSA and produced a single band at the same location in each experiment (Fig. 5A,C). Free probe was monitored on the gels, but due to differences in intensity have been deleted from the figure. The CRm resulted in an alternate band that did not migrate as far as the WT ABP E-box 2/CRE or mutated E-box 2 (Fig. 5B). More intense bands were detected when samples were incubated with the nuclear extract from the FSH (F) and db-cAMP (A) treated cells (Fig. 5). Band specificity was confirmed using excess unlabeled oligonucleotides resulting in the elimination of each specific band (Fig. 5, lane P). Immunoblot analysis indicated that the lower band seen in the WT E-box 2/CRE oligonucleotide EMSA (Fig. 5A) contains the class A bHLH E47 protein (Fig. 5D). This immunoblot of the EMSA of the wild-type E-box 2/CRE oligonucleotide confirmed the presence of bHLH protein in the transcriptional complex. Competition of EMSA bands was performed using excess unlabeled (cold) oligonucleotides (Fig. 6) to determine which class of transcription factor may be binding to the oligonucleotides. The radiolabeled and unlabeled oligonucleotides incorporated in the assays corresponded to the E-box 2/CRE (−108 to −133) region of the 619-bp (WT) ABP E-box 2/CRE (WT), mutated

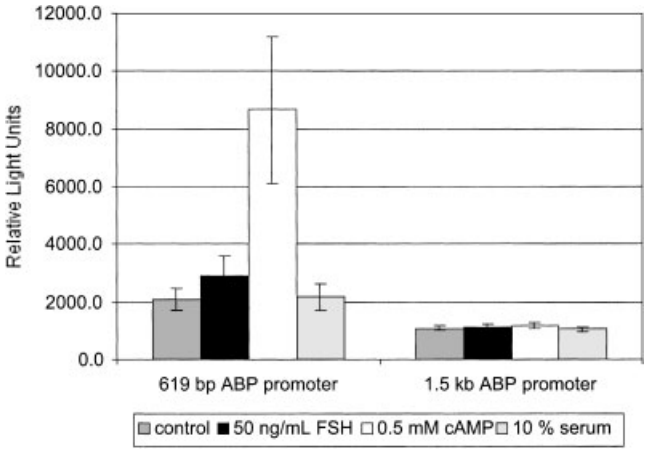

Fig. 2. Comparison between the full-length 1.5 kb and proximal 619-bp ABP promoter reporter construct. Primary cultured Sertoli cells were transfected with either the full-length or truncated form of the ABP promoter and left untreated (control) or treated with FSH, db-cAMP, or 10% serum. Cells were harvested at 48 hr. Data are presented as relative light units ± SEM of triplicate samples from a minimum of three different experiments.

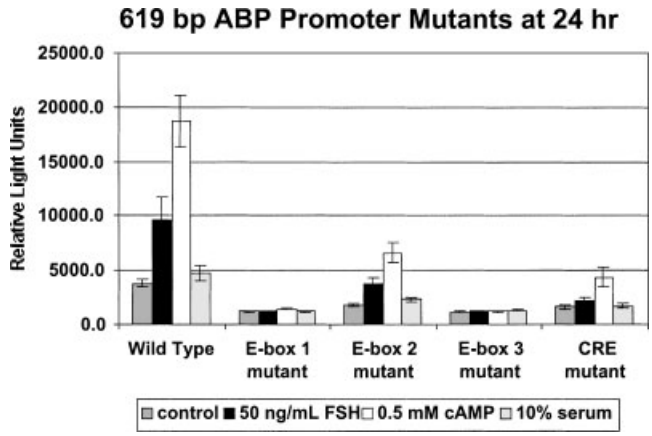

Fig. 4. Effect of mutations in the 619-bp ABP promoter on reporter gene expression. Primary cultured Sertoli cells were transfected with either the WT or mutant 619-bp ABP promoter constructs. Mutations were in the regions indicated in Figure 1. Cultures were untreated (control) or treated with FSH, db-cAMP, or 10% serum. The samples were harvested at 24-hr post-transfection and treatment. Data are presented as relative light units ± SEM of triplicate samples from at least three different experiments.

E-box 2 (EBm), CRm, consensus CRE (CRE), and transferrin CRE-like PRII (TCR). The null (N) lanes show binding in the absence of competing oligonucleotides. In the competition assay with the radiolabeled WT E-box 2/CRE (Fig. 6A), the unlabeled WT oligo and CRm completely inhibited binding to the WT oligo. TCR and consensus CRE partially inhibit the interaction. The mutated E-box 2 (EBm) inhibits the interaction to a lesser extent. These data suggest the DNA–protein interactions with the wild-type E-box 2/CRE response element occur primarily with the bHLH and E-box, although some of the DNA–protein interaction occurs between the DNA and CREB. In the competitive EMSA using the radiolabeled CRm (Fig. 6B), there is complete inhibition with only the unlabeled CRm and slight inhibition with the wild-type E-box 2/CRE sequence. Since all other unlabeled oligonucleotides contain only a CRE or modified CRE, this suggests no CREB binding, but only bHLH binding. Radiolabeled mutated E-box 2 (Fig. 6C) competed with unlabeled E-box 2/CRE (WT), CRm, and mutated E-box 2 (EBm). Partial inhibition with the transferrin PRII (TCR) and consensus CRE oligonucleotides was observed. Taken together, these results indicate that both CREB and bHLH are present and may interact in this wild-type ABP E-box 2/CRE region. However, further studies are needed to confirm CREB binding and data suggest the bHLH proteins show preferential binding to this site.

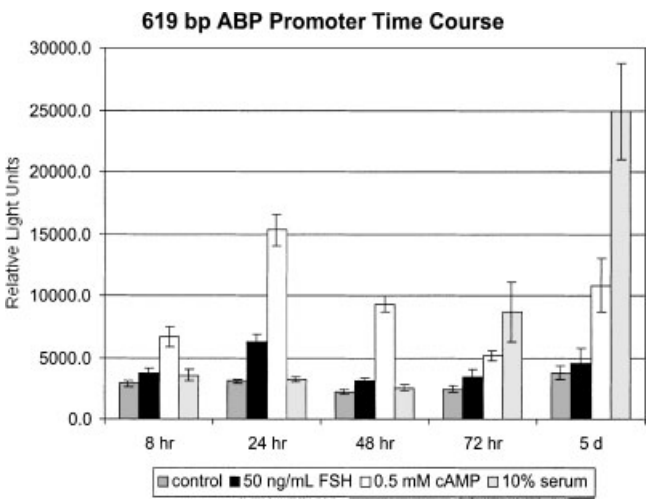

Fig. 3. Time course study to determine the stimulation of the proximal 619-bp ABP promoter construct. Primary Sertoli cell cultures were transfected with the 619-bp ABP promoter construct and left untreated (control), or treated with FSH, db-cAMP, or 10% serum. The duration of treatment prior to cell harvest is shown. Data are presented as relative light units ± SEM of triplicate samples from at least three different experiments.

To determine the functional significance of bHLH protein activation of the 619-bp ABP promoter, a series of co-transfections with a vector (pCI-neo) expressing human Id1 was performed. Id1 binds most bHLH proteins and inhibits binding to DNA such that bHLH actions are blocked. The pCI-neo expression vector alone was used as a control and should not inhibit the 619-bp ABP promoter. The pCI-neo plasmid expressing the bHLH inhibitory protein Id1 protein should inhibit the 619-bp ABP promoter as it is presumed that the E-boxes within this promoter act to bind bHLH. Observations suggest that overexpression of Id1 inhibits both FSH and db-cAMP induced ABP promoter activity compared to vector alone or control cells (Fig. 7). The overexpression of Id1 protein appears to inhibit endogenous bHLH proteins leading to inactivation of the 619-bp ABP promoter in comparison to the pCI-neo vector alone.

Observation with the ABP promoter suggested bHLH and E-box response elements are important for mediating FSH and cAMP actions, in addition to the CRE element. To further investigate this an artificial promo-

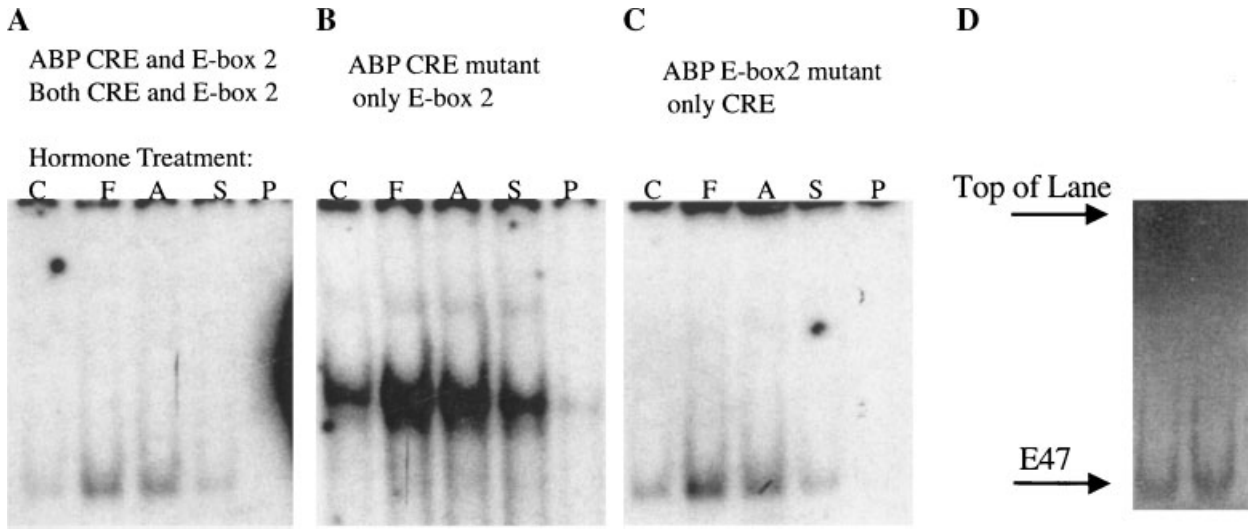

Fig. 5. Effect of cell treatments on transcription factor binding to the ABP promoter sequences. Electromobility shift assays using the following radiolabeled nucleotides were performed: E-box2/CRE ABP region containing both CRE and E-box 2 (**A**), ABP CRE mutant, only E-box 2 intact (**B**), and ABP E-box 2 mutant, only CRE intact (**C**). Nuclear extracts (5 μg) were obtained from untreated Sertoli cells (C) or Sertoli cells treated with FSH (F), db-cAMP (A), or 10% serum (S). Displacement was observed with excess cold oligonucleotide (P) of each respective band indicating band specificity. **D**: Immunoblot of EMSA gels the ABP E-box 1, and ABP E-box 2 were probed with antibody to the bHLH family member E47. The band observed indicates E47 bHLH protein binding to the ABP E-boxes (arrow). Data are representative of a minimum of three different experiments.

ter was designed that contained both a consensus E-box and CRE (Fig. 8). The consensus E-box and CRE regions were flanked by sequences lacking any known response elements. Restriction sites were engineered for ease of insertion into the luciferase reporter construct. The artificial promoter separates the E-box and CRE by 38 bp (Fig. 8). The basal level of activity for the artificial promoter was determined. A time course experiment indicated that FSH and db-cAMP stimulates the reporter gene starting at 8 hr and was optimally stimulated at 24 hr, and subsequently declined between 48 and 72 hr (data not shown). No stimulation was observed with serum alone. The control reporter plasmid not containing this artificial promoter had negligible basal activity and was not influenced by any treatment (data not shown). Mutations of the artificial promoter eliminated either the E-box or the CRE region (Fig. 9). The CRE (mutated E-box) promoter construct was stimulated by both FSH and db-cAMP, but not serum (Fig. 9B). Stimulation with FSH and db-cAMP was approximately 30% less than the WT control construct (Fig. 9A). The E-box (CRm) promoter was also stimulated by both FSH

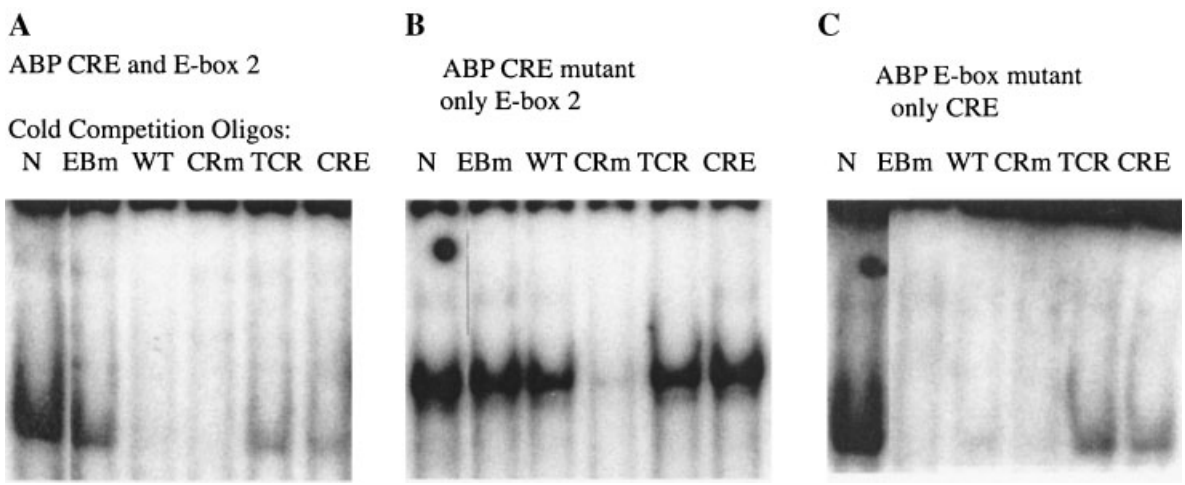

Fig. 6. EMSA competition assays indicate the presence of bHLH and CREB in the nuclear extract of db-cAMP treated Sertoli cells. Excess cold competing oligonucleotides were used that contained the WT ABP CRE/E-box 2 (WT), mutated ABP E-box 2 (EBm), mutated ABP CRE (CRm), transferrin CRE-like PRII (TCR), and consensus CRE (CRE). Reactions with no cold oligonucleotides were used as controls (N). **A**: The WT ABP CRE and E-box 2 promoter region. **B**: ABP CRE mutant. **C**: The ABP E-box 2 mutant. Data are representative of a minimum of three different experiments.

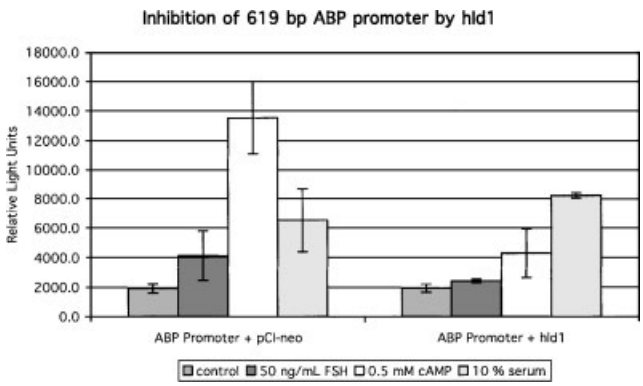

Fig. 7. Inhibition of 619-bp ABP promoter activity by the bHLH inhibitory factor, human Id1 (hId1). Primary Sertoli cells were co-transfected with the 619-bp ABP promoter construct and either pCI-neo or pCI-neo expressing hId1. Cultures of Sertoli cells untreated (control) or cells treated with FSH, db-cAMP, or 10% serum were harvested following a 24-hr incubation. Data are presented as relative light units ± SEM of triplicate samples from a representative experiment of a minimum of three different experiments.

and db-cAMP at approximately 5% the level of the WT construct (Fig. 9C). The basal control levels of this mutated construct were significantly lower than the other constructs. Observations demonstrate that both response elements are needed for optimal activation of the artificial promoter, but the CRE is the predominant response element contributing to promoter activation.

DISCUSSION

The ABP (i.e., SHBG) aids in the transport of steroids throughout the body and within the seminiferous tubule lumen to the epididymis. The transcriptional regulation of the ABP promoter in Sertoli cells has not previously been elucidated. However, DNA footprinting and deletion mapping of the ABP promoter have been performed (Fenstermacher and Joseph, 1997), yet specific response elements were not included in these studies. The current study investigates the E-box and CRE within the proximal 619 bp of the 1.5-kb ABP promoter and suggests that these are essential elements for ABP gene transcription. The 619-bp proximal promoter was used due

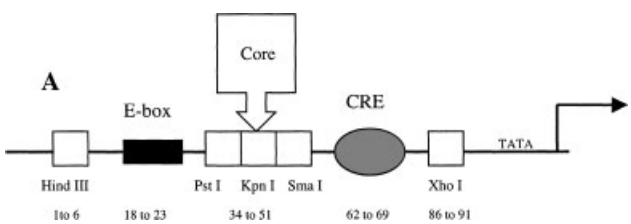

Fig. 8. Schematic drawing of the artificial promoter. There is an E-box located in the first cassette. The E-box was derived from the mouse transferrin promoter and the surrounding sequence contains no other known response elements. The second cassette contains a consensus CRE, also flanked by a sequence that contains no other known response elements. Located between the two cassettes is a region of three restriction sites with a core sequence containing no known response elements. There is a TATA box located prior to the luciferase gene in the pLuc-MCS plasmid.

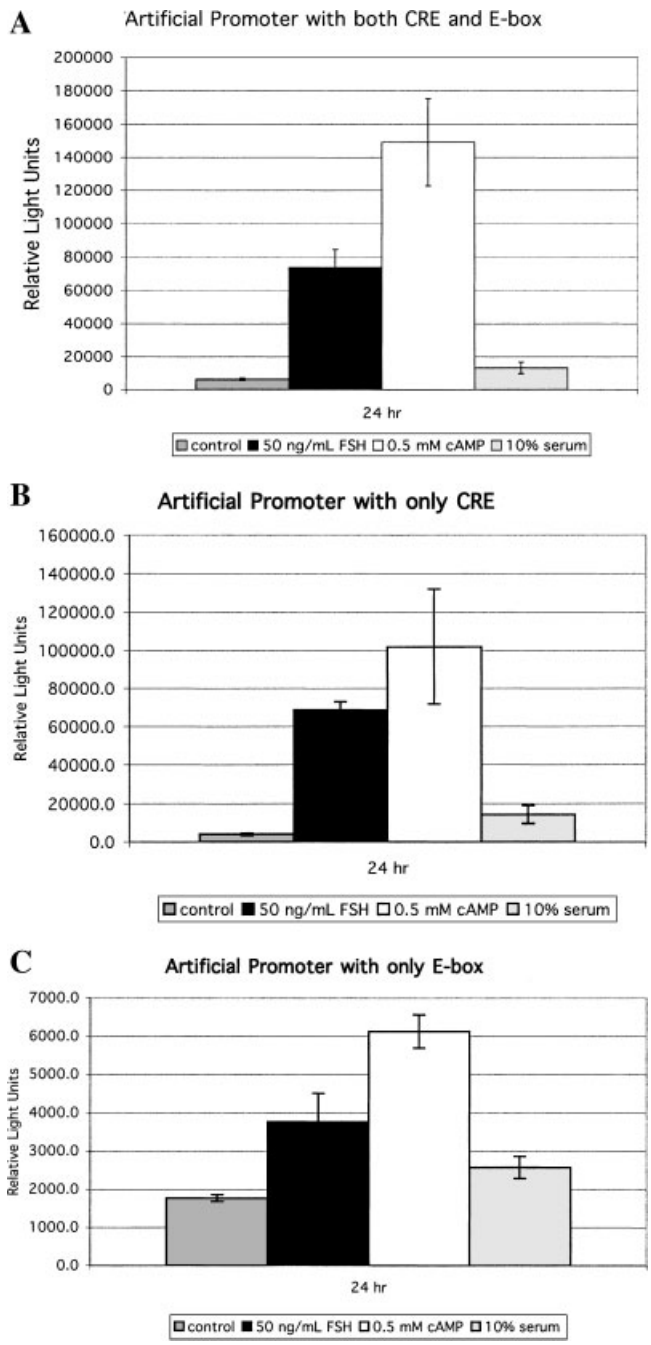

Fig. 9. Artificial promoter with both CRE and E-box (**A**) or mutated to contain either CRE (**B**) or E-box (**C**). Sertoli cells were transfected with the mutated artificial promoter to determine the effectiveness of CRE (CRE) or the E-box (E-box) alone. The Sertoli cells were untreated (control) or treated with FSH, db-cAMP, or 10% serum. The samples were harvested at 24-hr post-transfection. Data are presented as relative light units ± standard deviation of triplicate samples from a representative experiment of a minimum of three different experiments.

to the apparent presence of a repressor in the 1.5-kb promoter (Hall et al., 1990).

It was observed that the E-box 1 and E-box 3 within the proximal 619 bp of the ABP promoter are essential in the regulation of transcription. In contrast, E-box 2

mutations did not completely downregulate the ABP promoter. A possible explanation for this finding is that the CRE and E-box 2 overlap and retain activity in the absence of each other. Two base pairs at the 3′-end of E-box 2 overlap with the 5′-end of the CRE. The CRm was downregulated similarly to the E-box 2 mutation. This suggests that there may be interactions at this site, −129 to −118.

A cooperative mechanism at the E-box 2 and CRE site would suggest that one of the transcription factors would need to bind initially, thereby facilitating the binding of the second. This interaction would suggest that both transcription factors are necessary for optimal upregulation of the gene. While this hypothesis is novel and the transfection data partially support this idea, it is not supported in the EMSA experiments. The gel shifts indicate only one DNA–protein interaction in the WT promoter and immunoblotting suggest that the bHLH protein E47 is bound to the E-box 2. Conversely, a competitive mechanism may explain these results. Since the E-box 2 mutation partially alters the CRE, this may explain a reduced response. However, if the E-box were the primary binding site, it would be expected that this mutation would not result in downregulation. The gel shifts and immunoblots indicate that the WT ABP promoter most likely primarily binds a bHLH protein.

There are several similarities and differences in comparing the transferrin promoter and the ABP promoter. Both of these promoters are differentially regulated in two cell types, namely in hepatocytes and testicular Sertoli cells. The transferrin promoter is regulated by binding iron in hepatocytes (Idzerda et al., 1986) and through hormonal regulation in Sertoli cells (Chaudhary et al., 1997; Chaudhary and Skinner, 1999). The ABP promoter is regulated by hormones in both cell types. In the liver, SHBG is produced in response to estrogens and decreased in the presence of androgens (Anderson, 1974). ABP secretion in Sertoli cells is dependent on FSH and androgens (Hansson et al., 1975; Hall et al., 1990).

The response elements responsible for the stimulation of the transferrin and ABP promoters are similar. Both promoters have E-boxes and CRE or CRE-like (PRII) elements. In the transferrin promoter, the E-box and PRII are separated by 220 bp, or one turn of the DNA around a nucleosome, allowing the bHLH and CREB to come into close association (Chaudhary and Skinner, 1999). The ABP promoter has the same elements, but is regulated in a slightly different manner. The ABP promoter has two E-boxes separated by 227 bp and another E-box overlapping the CRE (Fig. 1). The two E-boxes may potentially have the same function as the E-box and PRII in transferrin. Although it is unclear at this time whether the bHLH of these two E-boxes (E-box 1 and E-box 3) interact directly or through an intermediary protein such as CBP. The overlapping E-box and CRE may function through an interaction exerting a second level of control for ABP gene transcription.

Observations with both the ABP promoter in the current study, and transferrin promoter in previous studies (Chaudhary and Skinner, 1999), suggest bHLH proteins and E-box elements are important for FSH and cAMP actions on Sertoli cells, in addition to the classic CRE and CREB factors. This suggests the classic FSH-mediated signal involving cAMP, CREB, and CRE is more complex and requires other factors to be involved. An artificial promoter containing both E-box and CRE was used to further investigate this phenomenon. The artificial promoter was designed to easily manipulate the relationship between the E-box and CRE. The time course data (data not shown) demonstrated an initial stimulation by FSH and db-cAMP at 8 hr and maximum stimulation at 24 hr and then declining. This is similar to the ABP and transferrin promoters. The serum response remains low at all time points. This phenomenon is not seen in the ABP and transferrin promoters. This indicates a potential indirect effect of the serum on the ABP and transferrin promoters through alternate response element(s). The deletion mutants show both E-box and CRE elements are required for optimal promoter activation. The CRE promoter is stimulated by both FSH and db-cAMP and induced the majority of promoter activation. The E-box artificial promoter is also stimulated by FSH and db-cAMP, but the basal level of activation is over 10 times less than the CRE construct. The observations with this artificial promoter are distinct from the ABP promoter and previous transferrin promoter data. A potential difference is that the E-box may need a second response element brought into close proximity through the binding of DNA around a nucleosome to be optimally active. This could be addressed through the addition of core sequences to increase the distance between the E-box and CRE (i.e., 200 bp). The observations with this artificial promoter suggest that the interactions between E-box and CRE elements involve more than simply both elements being present on the promoter.

Observations presented indicate that the overexpression of the bHLH inhibitory protein Id-1 blocks the hormone activation of the ABP promoter. Id proteins bind bHLH proteins preventing binding to DNA and block general bHLH actions. Observations demonstrate that bHLH proteins are essential for ABP transcriptional regulation in Sertoli cells. How the different E-box elements may interact will likely be important and requires further investigation. FSH and cAMP are important regulators of ABP promoter activation such that CREB and the identified CRE are speculated to be essential. Observations suggest that the E-box 2 and CRE interactions are complex and primarily involve bHLH binding and potential interactions with CREB. The role that the E-box 2 and CRE have in these interactions of bHLH and CREB requires further investigation. However, all the elements identified appear to be critical in ABP transcriptional regulation. The observations presented in the current study support previous observations with the transferrin promoter (Chaudhary et al., 1997; Chaudhary and Skinner, 1999) on the importance of bHLH proteins in the transcriptional activation of differentiated genes in Sertoli cells. The

classic concept that FSH activation of cAMP and subsequent activation of CREB and binding to CRE is not sufficient to explain Sertoli cell gene expression. Observations presented further support a critical role of the differentiation class of transcription factors, bHLH proteins, in the transcriptional regulation of Sertoli cell differentiation.

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