CREB Binding Protein (CBP) Activation Is Required for Luteinizing Hormone Beta Expression and Normal Fertility in Mice

Ryan S. Miller, Andrew Wolfe, Ling He, Sally Radovick and Fredric E. Wondisford

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Normal function of the hypothalamic-pituitary-gonadal axis is dependent on gonadotropin-releasing hormone (GNRH)-stimulated synthesis and secretion of luteinizing hormone (LH) from the pituitary gonadotroph. While the transcriptional coactivator CREB binding protein (CBP) is known to interact with Egr-1, the major mediator of GNRH action on the Lhb gene, the role of CBP in Lhb gene expression has yet to be characterized. We show that in the LβT2 gonadotroph cell line, overexpression of CBP augmented the response to GNRH and that knockdown of CBP eliminated GNRH responsiveness. While GNRH-mediated phosphorylation of CBP at Ser436 increased the interaction with Egr-1 on the Lhb promoter, loss of this phosphorylation site eliminated GNRH-mediated Lhb expression in LβT2 cells. In vivo, loss of CBP phosphorylation at Ser436 rendered female mice subfertile. S436A knock-in mice had disrupted estrous cyclicity and reduced responsiveness to GNRH. Our results show that GNRH-mediated phosphorylation of CBP at Ser436 is required for Egr-1 to activate Lhb expression and is a requirement for normal fertility in female mice. As CBP can be phosphorylated by other factors, such as insulin, our studies suggest that CBP may act as a key regulator of Lhb expression in the gonadotroph by integrating homeostatic information with GNRH signaling.

Reproductive viability in mice is dependent on normal function of the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GNRH) is produced by hypothalamic neurosecretory cells and released in a pulsatile manner into the hypothalamo-hypophyseal portal circulation, through which the hormone is transported to the anterior pituitary gland. GNRH bound to its receptor on pituitary gonadotrophs results in an increase in GNRH receptor density and stimulates the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the circulation (14, 30, 41). LH and FSH are heterodimeric glycoproteins consisting of a common β-subunit and hormone-specific α-subunit encoded by the Lhb and Fshb genes, respectively. Lhb and Fshb gene expression in the anterior pituitary is dependent on pulsatile GNRH secretion. Rapid, high-amplitude GNRH pulses stimulate an increase in Lhb mRNA levels, leading to an increase in LH synthesis and release from the gonadotroph (11, 18, 45, 51).

At least three major transcription factors are important for Lhb gene expression: steroidogenic factor 1 (SF-1), pituitary homeobox factor 1 (Pitx1), and early growth response factor 1 (Egr-1). GNRH stimulates the expression of Egr-1, resulting in Egr-1 binding to two conserved cis elements of the proximal Lhb promoter (5, 43, 49). Egr-1 is rapidly and markedly induced by GNRH, while SF-1 and Pitx1 expression levels are unchanged following GNRH administration (43, 49). In rat, Egr-1 expression increases in proestrus, suggesting that it may be an important signal for ovulation (39). Loss of Egr-1 in the gonadotroph, unlike loss of SF-1 or Pitx1, renders the cell unable to respond to GNRH, and Egr-1 knockout mice have a selective defect in LH expression that is not responsive to gonadectomy (13, 29). In contrast, pituitary gland-specific SF-1 knockout mice have markedly decreased expression of LH and FSH, but they are able to produce LH in the pituitary gland in response to exogenous GNRH (55). In the LβT2 gonadotroph cell line, mutation in the PITX binding site of the human Lhb promoter decreased basal, but not GNRH1-induced, transcriptional activity (13). These data indicate that while SF-1 and Pitx1 enhance the transcriptional response, Egr-1 is the major mediator of GNRH-stimulated Lhb gene expression.

CREB binding protein (CBP; Crebbp is the mouse gene transcript [mRNA] for CBP) and the closely related protein p300 have been identified as essential cofactors for many nuclear transcription factors. CBP was first identified as a transcriptional coactivator of CREB (7, 28). Protein kinase A (PKA) phosphorylation of CREB at Ser133 promotes the formation of a transcriptional complex on the cyclic AMP (cAMP) response element (CRE) containing CREB, CBP, and CREB-regulated transcription coactivator 2, resulting in activation of gluconeogenic genes in hepatocytes (36, 50). CBP is subsequently able to activate gene transcription via intrinsic histone acetyltransferase activity and recruitment of other transcription factors (7, 15, 16, 28, 33, 46). Conversely, insulin is able to induce PKC phosphorylation of CBP at Ser436, a residue located near the CRE binding domain of CBP (53). CBP phosphorylation at Ser436 disrupts the complex on the CRE, freeing CBP to interact with other transcription factors (21).

We chose to study CBP action in the gonadotroph because CBP can be phosphorylated by mitogen-activated protein kinase (MAPK) and PKC, both of which are direct pathways for CBP activation via GNRH and insulin (21, 24). It has been established that gonadotrophs express insulin receptors on the cell surface and that LβT2 cells are able to bind insulin (4, 17). We and others have demonstrated that insulin augments GNRH-mediated Lhb expression and LH secretion primarily via induction of Egr-1 (1, 4, 40). Thus, CBP may function to integrate multiple inputs, reproducible...
ductive and metabolic, to regulate Lhb gene expression (26). As prior studies have shown that Egr-1 and CBP can interact directly via the transactivation domain of Egr-1 and the N terminus of CBP to increase gene transcription, we hypothesized that CBP interacts with Egr-1 in the gonadotroph to promote Lhb gene expression (38). In current studies, we test the hypothesis that CBP activation via phosphorylation is required for synthesis of LHβ and for normal gonadotroph function. Our results indicate that GNRH-mediated phosphorylation of CBP is required for Egr-1-mediated Lhb gene expression and is necessary for normal function of the central reproductive axis.

MATERIALS AND METHODS

Constructs. The Lhb promoter-luciferase reporter contains the −140 to +1 fragment of the mouse Lhb gene promoter cloned into pALuc proximal to luciferase as previously described (4). The wild-type CBP and S436A mutant vectors were generated using the QuickChange site-directed mutagenesis kit (Stratagene) as previously described (56). The PatternDetect in vivo signal transduction pathway trans-reporting systems (Agilent Technologies) was used to generate a trans-activator protein consisting of Egr-1 fused to the Saccharomyces cerevisiae GAL4 DNA binding domain (DBD). The primers used to generate the Egr-1 insert were designed to achieve in-frame translation of the Gal4–Egr-1 fusion protein and to avoid the Egr-1 start codon. The primer sequences were 5′-GTCGGTGAATTCCTCACGCTGCAACGGCATTT and 5′-GTTCAGAGGGTTAGGGAGAGCA (sense) and 5′-GTGGTAATACCGAAGTATTAAGAGCCTTTAAGTCC (antisense). Sequencing confirmed the correct orientation of the insert in relationship to the Gal4 DBD.

Transfection. LjT2 cells were maintained in Dulbecco modified Eagle medium (DMEM; Cellgro) supplemented with 10% fetal calf serum. Cells were plated at approximately 1 × 10⁵ cells per well in a 6-well plate the night prior to transfection. For experiments that did not require viral transduction, cells were transfected using Lipofectamine 2000 reagent (Invitrogen) the morning after being split. Medium was changed the following day to DMEM with 0.56 mmol glucose and 10% charcoal-stripped fetal bovine serum (FBS). The following morning, cells were treated with 30 nM GNRH for the times indicated below prior to harvest. Cells were harvested in either lysis buffer for luciferase activity, cell lysis buffer (Cell Signaling) for protein assay, or TRIzol (Invitrogen) for RNA isolation. For measurement of luciferase activity, 100 μL of cell lysate was mixed with luciferin substrate and luminescence was measured with a Lumat LB luminometer (Berthold Technologies).

Viral transduction. For CBP knockdown, cells were transduced with adenovirus expressing short hairpin RNA (Ad-shRNA) against the 5′ untranscribed region (5′UTR) of Crebbp (sequence 1, 5′-CACCGTTGCTGAGGCTTCAGAATTGGCCTGAGCTGCAGCAC; sequence 2, 5′-AACAGTGCTGAGCTGAGTTTGTTCATCCGCCAACATCTCACCC; sequence 3, 5′-AACAGTGCTGAGCTGAGTTTGTTCATCCGCCAACATCTCACCC). For Egr-1 knockdown, cells were transfected with Ad-shRNA against a region of the coding sequence of Egr-1 (sequence 1, 5′-CACCGGGACTTAAAGGCTCTTAATACCGAAGTATTAAGAGCCTTTAAGTCC; sequence 2, 5′-AACAGTGCTGAGCTGAGTTTGTTCATCCGCCAACATCTCACCC). Virus was generated using the U6 promoter vector (Invitrogen) and verified by sequencing. Cells were transduced either 24 h after being plated or 24 h after transfection. Medium was changed 24 h later, and assays were performed 48 h after transduction.

One-hybrid assay. LjT2 cells were maintained as described above. The morning after being plated on 6-well plates, cells under all conditions were transduced with Ad-shRNA directed against the 5′UTR of CBP. The following day, cells were transfected with the plasmids indicated below using Lipofectamine 2000 according to the manufacturer’s protocol. All cells were cotransfected with pFA-CMV expressing the GAL4 DBD–Egr-1 fusion protein (Gal4–Egr-1) and the pFR-Luc reporter (Agilent). Cells were also transfected with either an empty vector, wild-type (WT) CBP, or S436A CBP, as indicated below. A subset of cells was transfected with the pFR-Luc reporter only and treated with GNRH to demonstrate a lack of nonspecific activation of the reporter. Six hours after transfection, cell culture medium was changed to DMEM containing 10% charcoal-stripped FBS. The following morning, cells were treated with 30 nM GNRH for 4 h prior to being harvested in lysis buffer for the luciferase assay performed as described above.

Quantitative PCR (Q-PCR). Following treatment with GNRH, cells were harvested in TRIzol reagent and RNA was isolated according to the manufacturer’s protocol. RNA was quantitated by spectrophotometry and reverse transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad). Reverse transcription-PCR (RT-PCR) was performed using the iQ SYBR green supermix on the MyQ single-color real-time PCR detection system module for the iCycler (Bio-Rad). RT-PCR was performed for Lhb, Egr-1, and Crebbp mRNA using 36b4 mRNA as a control. The following primers were used for RT-PCR: the Lhb forward primer 5′-AACCTCTGGCGCAGAGAAATG, the reverse primer 5′-CAGATCTCCGAGCACTGCTA, the Egr-1 forward primer 5′-CACCGGGCTCTCAATCTCAGAAG, the reverse primer 5′-TCTACCACCTGCCTTCCTCATT, the Crebbp forward primer 5′-CAGAGAAGGGCTTCTCTGAGCT, the reverse primer 5′-CTTGGTTAGGGTAAGGAGAGCA, the 36b4 forward primer 5′-TGTTAGAAAGGCCATT, and the reverse primer 5′-CCAGGAGCAAGTTGGAGTA.

ChIP. Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express kit (Active Motif) according to the manufacturer’s protocol. Following treatment with GNRH for the times indicated below, cross-linking was performed with formaldehyde and cells were harvested in lysis buffer. DNA was sheared by sonication, and chromatin was incubated with CBP antibody, Egr-1 antibody, or nonspecific IgG (Santa Cruz). After washing of cells and reverse cross-linking, PCR was performed using Taq polymerase (Denville) for the proximal Lhb promoter (positions −307 to −6) using the following primers: 5′-CTGAAAGGTTTCCAGATCTCCTCAGGCT (sense) and 5′-TCCAAAGGTCTTACCTTCCCTACCTTCTCTCTCC (antisense). The distal promoter (−1189 to −893) primers were 5′-AACAAAGTGGGGATCCTCTGGATCGG (sense) and 5′-GATATCCAGACCGTCTGCTGTTGG (antisense). PCR products were detected following electrophoresis on a 1% agarose gel. Q-PCR was performed as described above, using primers for the proximal Lhb promoter. Q-PCR data are corrected for input chromatin.

Western blot analysis. Proteins were resolved by SDS-PAGE on a 4 to 12% bis-Tris gradient gel (CBP) or 10% bis-Tris gel (Egr-1) and transferred to an Immobilon P membrane. Immunoblotting was conducted using commercially available polyclonal CBP and Egr-1 antibody (Santa Cruz) or affinity-purified rabbit anti-phospho-serine 436-CBP generated by Invitrogen (Carlsbad, CA) against the peptide PVCLPLKNA(pS)DKRNQQTIL (pS indicates phosphoserine) as previously reported (23). Blots were incubated with antibody overnight at 4°C, and antibody binding was visualized using the ECL Plus reagent (Amer sham Pharmacia, Piscataway, NJ).

Animal experiments and procedures. Animal protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. The generation of CBP S436A mice has been described previously (56). For breeding studies, 2- to 3-month-old mice were observed over 60 days. Pups were removed and counted the day after delivery. Vaginal cytology was assessed daily between 9 and 10 a.m. for the numbers of days indicated below in cycling female mice that were individually housed starting 7 to 10 days prior to study. Vaginal cells were collected with calcium alginate swabs soaked in 0.9% saline and dried on a slide. Cells were fixed in methanol and stained with the Diff-Quick kit (IMEB, Inc.) according to the manufacturer’s protocol. Stages were assessed according to predominant cell type (32). Blood was collected via mandibular bleed in the morning for basal samples or 8 to 9 p.m. for surge values. Serum LH and FSH levels were analyzed on the LumineX 2000IS platform using the Milliplex rat pituitary panel (Millipore). Serum testosterone levels were analyzed by the Ligand Assay Core at the University of Virginia. Ovaries and testes were collected from mice following CO2 as-
phyxiation and perfusion with Bouin’s solution (Sigma). Ovaries were sectioned every 100 μm and stained with hematoxylin and eosin by the Johns Hopkins University Molecular and Comparative Pathobiology Phenotyping Core. Gonadectomies were performed on 3- to 4-month-old male mice under ketamine-xylazine anesthesia. Blood was collected 10 days later for LH and FSH.

**Data analysis.** Results of RT-PCR are expressed as fold changes in gene expression from that of an untreated control. ChIP Q-PCR data are displayed as fold enrichment versus an untreated control. Statistical analyses were performed using the Student t test for comparisons between two groups. One-way analysis of variance (ANOVA) with the Student-Newman-Keuls post test was performed for all other comparisons. Statistical analyses were performed using GraphPad InStat version 3.0 for Windows NT (GraphPad Software, San Diego, CA). Differences were considered significant at a P of < 0.05.

**RESULTS**

The transcriptional coactivator CBP is required for GNRH-stimulated Lhb promoter activity in LβT2 cells. We utilized the LβT2 mouse-derived gonadotroph cell line to determine if CBP was required for GNRH-mediated activation of the Lhb promoter. Cells were cotransfected with a mouse Lhb promoter-luciferase reporter construct and either an empty vector (pcDNA3.1) or pcDNA containing full-length CBP. Overexpression of CBP did not significantly change basal reporter activity but did result in greater GNRH-stimulated activity than the empty vector (Fig. 1A). Fold change in Lhb promoter activity was affected as well, as overexpression of CBP resulted in a 3.9-fold increase in reporter activity following treatment with GNRH, versus a 2.5-fold increase in reporter activity in cells transfected with the empty vector (P < 0.05).

As CBP overexpression was able to enhance the response to GNRH, we next eliminated endogenous CBP to determine if this would decrease responsiveness to GNRH. LβT2 cells were transduced with adenovirus expressing either a scrambled shRNA or shRNA against the 5’ untranslated region of CBP. Cells that were transduced with shRNA against CBP had no response to GNRH, while cells transduced with scrambled shRNA demonstrated a 2.5-fold increase in LHβ mRNA following treatment with GNRH (Fig. 1B). Western blotting demonstrated a nearly total knockdown of endogenous CBP in cells transduced with viral shRNA against the 5’UTR of CBP (Fig. 1C).

CBP activation of the Lhb promoter is dependent on CBP phosphorylation at Ser436. The above-described experiments demonstrate that in the LβT2 gonadotroph cell line, CBP is required for GNRH-mediated stimulation of Lhb expression. To determine the mechanism by which GNRH activates CBP, changes in the quantities of Lhb, Egr-1, and Crebbp mRNA were measured by real-time RT-PCR following treatment of LβT2 cells

**FIG 1** CBP mediates GNRH induction of Lhb expression in LβT2 cells. (A) Luciferase activity was measured following GNRH treatment in cells transfected with an Lhb promoter-luciferase reporter and either pcDNA3.1 only or pcDNA containing full-length CBP. RLU, relative light units. (B) Relative levels of Lhb mRNA expression were determined in LβT2 cells that were transfected with either scrambled Ad-shRNA or CBP Ad-shRNA and then treated with GNRH 48 h later. (C) Immunoblot of total CBP from LβT2 cells transfected with either scrambled (Scr) or CBP Ad-shRNA. (D to F) Changes in transcript levels of Lhb, Egr, and Crebbp were determined by immunoblotting following GNRH treatment for the indicated periods of time. (H) Immunoblot of phospho-CBP with and without alkaline phosphatase. Means at points without a common letter differ (P < 0.05). *, P < 0.01. Bars represent means ± standard errors of the means (SEM).
with GNRH. We found that while Egr-1 mRNA increased over 50-fold and Lhb mRNA increased nearly 3-fold, there was no change in the level of Crebbp mRNA following treatment with GNRH (Fig. 1D to F).

We then sought to determine if GNRH activated CBP by phosphorylation. Phosphorylation of CBP at Ser436 has been shown to play an important role as a regulator of metabolism. We previously reported that insulin regulates hepatic gluconeogenesis through CBP phosphorylation at Ser436 (21, 56). To determine the role of CBP phosphorylation in the gonadotroph, we isolated the antibody for the site phosphorylated by GNRH. By probing with an antibody specific to phosphorylated CBP or the mutant S436A CBP. Cells were treated with GNRH 4 h prior to the harvest of RNA. *, P < 0.01 versus values for WT untreated, pcDNA-transfected, and mutant untreated cells; #, P < 0.05. Bars represent means ± SEM.

To determine if CBP phosphorylation at Ser436 is required for GNRH stimulation of LHB promoter activity, LβT2 cells were transfected with either scrambled shRNA or CBP 5’ UTR Ad-shRNA, followed by transfection with pcDNA3.1 only, pcDNA expressing WT CBP (CBP-wt), or pcDNA expressing S436A mutant CBP/CBP-mut). (B) Relative Lhb transcript levels in LβT2 cells following transduction with adenovirus expressing CBP 5’UTR shRNA and transfection with pcDNA3.1, WT CBP, or S436A mutant CBP. Cells were treated with GNRH for 30, 60, or 90 min following treatment with GNRH, there was increased binding of CBP and Egr-1 to the region of the promoter containing the proximal GNRH-responsive elements (Fig. 3A). The distal region of the promoter does not contain Egr-1 binding sites and showed no increase in levels of bound CBP or Egr-1 following GNRH administration (Fig. 3A). This was also demonstrated by performing Q-PCR on chromatin samples using primers that span the Lhb promoter region containing the proximal Egr-1 binding sites. We detected an increase in Egr-1 binding at 60 and 90 min and an increase in CBP binding at 90 min after GNRH treatment (Fig. 3B).

We then sought to determine if the increase in CBP binding to the Lhb promoter reflected a CBP–Egr-1 interaction occurring in response to CBP phosphorylation but independent of changes in Egr-1 level. To detect GNRH-mediated interactions between CBP and Egr-1, we performed a modified mammalian two-hybrid assay with the full-length Egr-1 cDNA fused to the Gal4 DNA binding domain and a reporter construct containing the Gal4 consensus binding site (pFR-Luc). After knockdown of endogenous CBP with shRNA, cells were transfected with vectors expressing either WT or S436A mutant CBP. Following treatment with GNRH, cells transfected with WT CBP stimulated greater reporter activity than cells transfected with the empty vector or a vector expressing S436A mutant CBP (Fig. 3C). Cells transfected with only the pFR-Luc reporter showed only background-level activity. These results indicate that CBP activation of Egr-1 requires CBP phosphorylation at Ser436 but does not require an increase in Egr-1 synthesis (Fig. 3D).

The proximal Lhb promoter contains response elements to other transcriptional promoters that could also interact with CBP. We conducted a ChIP experiment after depleting LβT2 cells of Egr-1 to determine if CBP binding to the proximal Lhb promoter is lost in the absence of Egr-1. LβT2 cells were first transfected with Ad-shRNA against Egr-1 and then treated with GNRH prior to being cross-linked. Q-PCR was then performed using primers to amplify the proximal Lhb promoter as described above. In cells transfected with scrambled shRNA, Egr-1 and CBP binding to the proximal promoter increased following GNRH treatment. In contrast, CBP binding to the proximal promoter did not increase in cells transfected with Egr-1 Ad-shRNA (Fig. 3E). Immunoblotting was performed on whole-cell lysate protein samples obtained transfected with WT CBP showed a 2-fold increase in LHB mRNA following treatment with GNRH, while cells transfected with the empty vector or S436A CBP showed no response to GNRH (Fig. 2B). This shows that in LβT2 cells, GNRH-mediated Lhb expression is dependent on phosphorylation of CBP at Ser436.

CBP interactions with Egr-1 on the proximal Lhb promoter following GNRH treatment are phosphorylation dependent. Previous studies have shown direct interactions between CBP and Egr-1 in other systems (38, 44). As Egr-1 is critical to Lhb expression, we performed a chromatin immunoprecipitation (ChIP) assay to investigate the hypothesis that GNRH activation of CBP results in recruitment of CBP to the proximal region of the Lhb promoter containing Egr-1 binding elements. LβT2 cells were treated with GNRH for 30, 60, or 90 min followed by protein-DNA cross-linking. Following shearing and immunoprecipitation with antibodies against Egr-1 or CBP, PCR was performed on chromatin using a series of overlapping primers to detect binding across the proximal 1,500 bp of the promoter. After 30 min of treatment with GNRH, there was increased binding of CBP and Egr-1 to theregion of the promoter containing the proximal GNRH-responsive elements (Fig. 3A). The distal region of the promoter does not contain Egr-1 binding sites and showed no increase in levels of bound CBP or Egr-1 following GNRH administration (Fig. 3A). This was also demonstrated by performing Q-PCR on chromatin samples using primers that span the Lhb promoter region containing the proximal Egr-1 binding sites. We detected an increase in Egr-1 binding at 60 and 90 min and an increase in CBP binding at 90 min after GNRH treatment (Fig. 3B).

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from LßT2 cells 48 h after transduction with Egr-1 AD-shRNA to demonstrate target knockdown (Fig. 3F). These results indicate that GNRH-mediated recruitment of CBP to the proximal promoter requires Egr-1 and suggest that CBP interacts directly with Egr-1 on the Lhb promoter to promote Lhb transcription.

The S436A mutation in vivo results in impaired fertility in female mice. To determine the importance of CBP phosphorylation in vivo, breeding studies were performed with mice containing a generalized S436A knock-in mutation. Breeding pairs were established between WT and S436A mice to assess litter size and intervals between litters. While results of pairings between WT female and S436A male mice did not differ from those of WT × WT pairings, pairings consisting of a WT male and a S436A female resulted in significantly fewer pups per litter and longer times between litters than WT × WT pairings (Fig. 4A and B). Pairings with an S436A female had fewer litters over the course of 60 days, as demonstrated in Fig. 4C. This indicates that loss of CBP phosphorylation affects fertility in female mice but not in male mice.

S436A mice have abnormal cycling and a reduction in peak LH levels. To assess further the reproductive defect due to the loss of CBP phosphorylation, estrous cyclicity was evaluated in female WT and S436A mice. Vaginal swabs were collected on 16 consecutive days and stained for cells indicative of cycle phase. Representative cycling data are shown for two WT and two S436A female mice (Fig. 5A). Evaluation of ovarian histology by hematoxylin and eosin staining showed significantly fewer corpora lutea and more atretic follicles in S436A mice, indicating a disturbance in cycling and ovulation as a consequence of the mutation in CBP (Fig. 5B). S436A mice did not cycle normally; they spent a significantly greater proportion of time in diestrus and less time in estrus than WT mice (Fig. 5C). Cycling data were then collected in the morning on 5 consecutive days, and blood was collected in the evening for LH and FSH levels during diestrus and proestrus to capture gonadotropin levels during the LH surge in proestrus. While LH levels in diestrus were similar among the two groups, S436A mice did not have a significant increase in LH levels in proestrus versus diestrus, and the mean LH value in proestrus was

FIG 3 CBP–Egr-1 interactions on the proximal Lhb promoter in response to GNRH. (A, B) CBP and Egr-1 occupancy of the Lhb promoter in LßT2 cells was detected by chromatin immunoprecipitation following GNRH treatment for the indicated periods of time. PCR (A) and Q-PCR (B) were performed using primers against both the proximal (~126 to ~26) and the distal (~1189 to ~893) Lhb promoter. *, P < 0.01 versus untreated cells. (C) LßT2 cells were transduced with AD-shRNA against the 3′ UTR of CBP, followed by transfection with the pFR-Luc reporter. Cells were also transfected as indicated with pcDNA only, WT CBP, or S436A CBP. Luciferase (Luc) activity was then measured after treatment with GNRH. *, P < 0.01 versus values for WT untreated cells and other conditions; #, P < 0.05. (D) Model of postulated CBP–Egr-1 interactions in the modified two-hybrid system. (E) CBP and Egr-1 occupancy of the Lhb promoter in response to GNRH was detected by chromatin immunoprecipitation in LßT2 cells 48 h after Egr-1 knockdown with AD-shRNA. Real-time quantitative PCR was performed using primers against the proximal (~126 to ~26) Lhb promoter. *, P < 0.01. (F) Immunoblot showing Egr-1 protein following transduction of LßT2 cells with scrambled or Egr-1 AD-shRNA. Bars represent means ± SEM.
significantly higher in WT mice than in S436A mice (15.7 ng/ml versus 3.4 ng/ml; \( P < 0.01 \)) (Fig. 5D). In contrast, FSH levels in diestrus were similar among WT and S436A mice, and both groups demonstrated similar increases in FSH on the day of proestrus (Fig. 5E).

Our investigations of the S436A mutation in mice also demonstrated a defect in GNRH responsiveness in males, despite apparent normal fertility. In the basal state, male S436A mice did not exhibit abnormalities. Morning LH, FSH, and testosterone levels did not differ between WT and S436A male mice (Fig. 6A), and in S436A mice, testicular histology appeared grossly normal (Fig. 6B). To determine if male S436A mice respond normally to GNRH, we measured basal serum LH and FSH levels in mice that had been gonadectomized 10 days prior. In gonadectomized mice, FSH levels were not different, but LH levels were significantly higher in WT than in S436A mice (Fig. 6C). LH was then measured before and 10 min after subcutaneous administration of GNRH to intact males. While there was a 6.6-fold increase in LH in WT mice, we detected only a 1.3-fold increase in LH in S436A mice (Fig. 6D). FSH did not increase following GNRH administration in either WT or S436A mice. These data indicate that loss of CBP phosphorylation results in a defect at the pituitary level that cannot be overcome by GNRH stimulation.

**DISCUSSION**

In this report, we show that the transcriptional coactivator CBP is critical to Egr-1 mediated activation of Lhb expression in pituitary gonadotrophs and plays a critical role in reproductive function in female mice. In vitro studies were conducted in LβT2 gonadotrophs, as these cells have been used extensively to determine mechanisms of Lhb gene regulation (12, 27, 34, 37, 42, 45, 48). The proximal Lhb promoter contains an enhancer region with two Egr-1 binding sites and two SF-1 binding sites separated by a Pitx1 binding site (19, 25, 47). In LβT2 cells, one transcriptional coactivator, small nuclear RING finger protein (SNURF), has been shown to interact with Sp1 (found in the distal enhancer region) and SF-1 but not Egr-1 (8).

CBP functions as a coactivator for many transcription factors and can be phosphorylated by a number of different signaling pathways (21, 24, 46). While CBP and the related protein p300 have overlapping functions and high sequence homology, p300 lacks the consensus PKC phosphorylation site, conferring unique functions to the two proteins. Mice heterozygous for either a CBP or p300 knockout are viable, but homozygotes are embryonic lethal, as are CBP p300 compound heterozygotes (16). There is considerable evidence that CBP and p300 are present in cells at limiting concentrations, providing a potential mechanism for tight regulation of Lhb expression (22, 35). In gonadotrophs, GNRH signaling via PKA phosphorylation results in CREB phosphorylation. This event may recruit CBP to the CRE on the Cga gene promoter in rodents but not in humans, as the CRE is not present in the proximal human Cga promoter (37). According to our model, GNRH acting via PKC signaling pathways activates CBP via phosphorylation, allowing CBP to interact with Egr-1 on the Lhb promoter, thus permitting expression of Lhb (Fig. 7).

While our studies have not determined the exact mechanism by which CBP phosphorylation promotes interaction with Egr-1, a previous study using CBP protein fragments fused to GAL4 in a two-hybrid system showed that the N terminus of Egr-1 was able...
to physically interact with both the N and C termini of CBP, including the region containing Ser436 (38). Nuclear magnetic resonance (NMR) structure studies suggest a potential mechanism by which CBP phosphorylation allows the protein to interact with transcription factors. The TAZ1 domains of CBP and p300, which share 92% sequence identity, contain a serine at position 436 in CBP, versus a glycine at position 422 in p300. The fourth /H9251-H9252/helix of the TAZ1 domain was found to be longer in a CBP-transcription factor complex, owing to the presence of the helix-destabilizing glycine residue at the end of the p300 TAZ1 domain (9). Thus, CBP phosphorylation in this domain may alter the protein conformation in a way that would allow it to interact with transcription factors such as Egr-1.

Using complementary methods of CBP overexpression and knockdown via shRNA, we demonstrated that CBP is required for GNRH-mediated Lhb expression in the L/H9252/T2 cell line. This is consistent with the results of other studies that have identified CBP as an important regulator of pituitary glycoprotein hormone subunit genes. In GH cells, CBP was shown to be involved in cellular processes. We also showed that CBP is required for GNRH-mediated Lhb expression. We found that, while basal levels were unaffected by CBP knockdown, treating L/H9252/T2 cells with GNRH resulted in a rapid phosphorylation at Ser436. In order to test the functional significance of the serine phosphorylation site, we studied GNRH responsiveness in L/H9252/T2 cells expressing either WT or S436A mutant CBP. Initial experiments did not indicate a difference in GNRH responsiveness between WT and S436A mutant CBP. This is possibly because in cells transfected with mutant CBP, enough endogenous CBP was present to permit normal functioning of the cell. This is also consistent with results of prior studies that suggest that CBP can signal efficiently in limited concentrations (7, 26). By transducing cells with viral shRNA directed against the 5' UTR of CBP prior to overexpression, we were able to eliminate endogenous CBP while preserving WT and S436A transcripts produced by the pcDNA vector. Using this approach, we were able to demonstrate that while basal levels were unaffected by

FIG 5 Assessment of the reproductive phenotype of S436A mice. (A) Vaginal cytology was performed at 24-h intervals by microscopic evaluation of stained vaginal smears, and the predominant cell type was recorded for each day. Data are shown for two representative animals from each group. (B) Hematoxylin- and eosin-stained histological sections of ovaries are shown from wild-type and S436A knock-in mice. Scale bars, 200 mm. (C) The proportion of time in diestrus versus estrus was recorded for each group. There were 7 to 8 mice per group. (D, E) Evening serum LH (D) and FSH (E) levels were measured in diestrus or proestrus for WT or S436A mice. There were 4 to 6 mice per group. *, P < 0.01; #, P < 0.05. Bars represent means ± SEM.
the loss of WT CBP, cells expressing the S436A mutant CBP are unable to increase Lhb transcription in response to GNRH.

Our studies also demonstrate that CBP likely promotes Lhb expression via interactions with Egr-1, the most critical transcription factor for Lhb. We identified Egr-1 as a target of CBP coactivation for several reasons. First, CBP has been shown to directly interact with Egr-1. CBP overexpression increases Egr-1-mediated activation of a 5-lipoxygenase reporter construct in COS cells; conversely, Egr-1 can also activate CBP, resulting in acetylation and stabilization of Egr-1 (38, 52). Additionally, both CBP and Egr-1 are activated by PKC. PKC activation has been shown to increase Egr-1 expression levels in gonadotrophs following GNRH administration (5, 19), while atypical PKC has been shown to phosphorylate CBP in hepatocytes following administration of insulin (21). Consistent with studies showing CBP–Egr-1 interactions, we found that treatment of L/H9252T2 cells with GNRH increases binding of CBP to the proximal Lhb promoter in the region containing Egr-1 binding sites. This promoter region also contains binding sites for other transcription factors, including a response element for SF-1, which is known to interact with CBP. However, we showed that CBP was not able to bind the proximal Lhb promoter after Egr-1 knockdown. Thus, while it is possible that CBP interacts with other transcription factors on the Lhb promoter, CBP is not able to bind the promoter without Egr-1. This underscores the critical role for Egr-1–CBP interaction on the proximal Lhb promoter in response to GNRH.

We also found that phosphorylation of CBP at Ser436 was critical for Egr-1 activation, as we showed in a modified two-hybrid system that treatment of cells with GNRH resulted in activation of Egr-1 in cells expressing WT CBP, but not in cells expressing S436A mutant CBP. This experiment showed that CBP activated Egr-1 via interactions on the promoter, rather than by increasing Egr-1 mRNA levels, as the quantity of Egr-1 in this system remains fixed.

In the present study, we extended our findings from LBT2 cells and demonstrated the requirement of CBP for gonadotroph function in vivo. While the S436A mutation impairs fertility only in female mice, we detected a lack of GNRH responsiveness in both males and females. Breeding pairs containing an S436A mouse had both fewer litters over time and fewer pups per litter. In contrast, breeding pairs containing an S436A male exhibited normal fertility. This was not entirely surprising, as Lee et al. previously demonstrated female infertility but not male infertility in Egr-1 knockout mice (29). The reduction in litter size seen with S436A mice paired with normal fertility in male mutant mice suggests that there is a threshold of Lhb expression required for normal gonadal function.

We then tested peak LH responses in males and females and measured estrous cycling in females to determine if there was ad-
equate LH secretion to produce a proestrus surge. While S436A mice did exhibit estrous cyclicity, they spent more time in diestrus and had fewer proestrus surges than WT mice. This is consistent with our findings that S436A female mice can produce offspring, but with greater intervals between litters. Even so, the comparison of times between litters underestimates the extent to which fertility is impaired in S436A female mice, as several mice in the study never produced a second litter. Serum LH measurements showed that S436A mice were able to increase LH levels in proestrus but to a much lower magnitude than WT mice. Lower LH levels in proestrus may result in the release of fewer eggs per cycle, which is also consistent with our finding of reduced litter size. Ovarian histology corroborates these data, as ovaries from S436A mice have fewer corpora lutea and more atretic follicles than WT mice, indicating follicle formation without ovulation. Biochemically, our findings in male S436A mice were analogous to findings in female mice in that male S436A mice had a subnormal response to GNRH. As fertility and testicular histology were normal, this suggests that male mice have a lower threshold for LH levels needed for normal reproductive function. Our biochemical evaluation of S436A mutant mice also showed that the mutation did not alter baseline FSH levels or the FSH response to GNRH, thus emphasizing the specificity of the effects of the mutation to Lhb. Taken together, findings for male and female S436A mice indicate a defect specific to Lhb, specifically at the level of the pituitary gland, as S436A mice were unable to respond normally to GNRH.

Our findings are the first to identify the critical role of CBP in gonadotroph function. As a transcriptional coactivator, CBP can be activated by a number of different signaling pathways, including MAPK and PKC. As such, CBP may act as an integrator of signals in the gonadotroph, providing an additional level of responsiveness in the pituitary gland. Our group has previously shown that CBP can be phosphorylated in response to insulin at Ser436 via atypical PKC signaling (21, 53, 56). Phosphorylation of CBP via insulin results in dissociation of CBP from the CREB-CBP-TORC2 complex, and in gonadotrophs, it results in recruitment to the Lhb promoter and activation of Lhb gene expression. We and others have shown that insulin can increase GNRH-mediated Lhb expression and secretion with some evidence of a stimulatory effect of insulin and GNRH in LBT2 cells via activation of AKT and extracellular signal-regulated kinase (ERK) (1, 4, 10, 31, 40). This suggests that energy-homeostatic information can be integrated at the level of the pituitary gland, possibly with gonadotropin-releasing hormone and follicle-stimulating hormone release by cultured pituitary cells. Endocrinology 108:1441–1449.


2. Reference to a previous study, we showed that obese, hyperinsulinemic mice

3. Haisenleder DJ, Dalkin AC, Ortolano GA, Marshall JC, Shupnik MA.


