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Repression of the Inhibin α-Subunit Gene by the Transcription Factor CCAAT/Enhancer-Binding Protein-β

Anna D. Burkart, Abir Mukherjee, Esta Sterneck, Peter F. Johnson, and Kelly E. Mayo

Department of Biochemistry, Molecular Biology and Cell Biology, and Center for Reproductive Science, Northwestern University (A.D.B., K.E.M.), Evanston, Illinois 60208; Reproductive Endocrine Unit, Massachusetts General Hospital, Harvard Medical School (A.M.), Boston, Massachusetts 02114; and Molecular Mechanisms in Development Group, Laboratory of Protein Dynamics and Signaling, Center for Cancer Research, National Cancer Institute (E.S.), and Eukaryotic Transcriptional Regulation Section, Laboratory of Protein Dynamics and Signaling, National Cancer Institute (P.F.J.), Frederick, Maryland 21702-1201

Inhibin is a dimeric peptide hormone produced in ovarian granulosa cells that suppresses FSH synthesis and secretion in the pituitary. Expression of inhibin α- and β-subunit genes in the rodent ovary is positively regulated by FSH and negatively regulated after the preovulatory LH surge. We have investigated the role of the transcription factor CCAAT/enhancer-binding protein-β (C/EBPβ) in repressing the inhibin α-subunit gene. C/EBPβ knockout mice fail to appropriately down-regulate inhibin α-subunit mRNA levels after treatment with human chorionic gonadotropin, indicating that C/EBPβ may function to repress inhibin gene expression. The expression and regulation of C/EBPβ were examined in rodent ovary, and these studies show that C/EBPβ is expressed in ovary and granulosa cells and is induced in response to human chorionic gonadotropin. Transient cotransfections with an inhibin promoter-luciferase reporter in a mouse granulosa cell line, GRMO2 cells, show that C/EBPβ is capable of repressing both basal and forskolin-stimulated inhibin gene promoter activities. An upstream binding site for C/EBPβ in the inhibin α-subunit promoter was identified by electrophoretic mobility shift assays, which, when mutated, results in elevated inhibin promoter activity. However, C/EBPβ also represses shorter promoter constructs lacking this site, and this component of repression is dependent on the more proximal promoter cAMP response element (CRE). Electrophoretic mobility shift assays show that C/EBPβ effectively competes with CRE-binding protein for binding to this atypical CRE. Thus, there are two distinct mechanisms by which C/EBPβ represses inhibin α-subunit gene expression in ovarian granulosa cells. (*Endocrinology* 146: 1909–1921, 2005)

THE SECRETION OF various steroid and peptide hormones within the hypothalamic-pituitary-gonadal axis controls normal ovarian function in the rodent. Within the ovary are follicles at various stages of maturation awaiting signals to undergo growth and differentiation (1). A variety of hormones tightly regulate ovarian follicular development, and they do so by modulating ovarian gene expression. Many distinct patterns of gene expression are orchestrated by the gonadotropins FSH and LH in the rodent ovary (2). One subset of ovarian genes is initially positively regulated by FSH and subsequently negatively regulated by LH. This common gene expression pattern is shared by the aromatase (3), FSH receptor (FSH-R) (4, 5), LH receptor (LH-R) (4–6), estrogen receptor β (7), protein kinase A regulatory subunit RIIβ (8), and inhibin α- and β-subunit genes (9, 10).

Inhibin is a peptide hormone formed by the heterodimerization of the inhibin α-subunit with one of the two related β-subunits that also can homodimerize to form activin. The predominant site for inhibin synthesis in the female is within the granulosa cells of developing ovarian follicles (9, 10). Inhibin acts directly on the pituitary to suppress the synthesis and secretion of FSH (11). After the midcycle LH surge in the rodent, both inhibin subunits are rapidly down-regulated. This suppression of dimeric inhibin is permissive for the secondary FSH surge, which recruits a new cohort of follicles and begins another reproductive cycle, thereby maintaining cyclicity (11–13).

Signaling through both the FSH-R and LH-R involves, at least in part, G protein-mediated activation of adenylyl cyclase and cAMP production (14–16). Higher levels of cAMP are generated by LH in preovulatory follicles than by FSH in smaller preantral follicles (17–19); therefore, it is likely that different transcription factors are activated by FSH and LH. Members of the basic leucine zipper (bZIP) family of transcription factors, named for the bZIP motifs in their carboxyl-terminal regions that confer their DNA-binding and dimerization abilities (20–22), have been implicated in the transcription of cAMP-responsive ovarian genes. Among the bZIP proteins are the activating transcription factors

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Abbreviations: ATF, Activating transcription factor; bZIP, basic leucine zipper; C/EBPβ, CCAAT/enhancer-binding protein-β; CRE, cAMP response element; CREB, cAMP response element-binding protein; DTT, dithiothreitol; FSH-R, FSH receptor; hCG, human chorionic gonadotropin; Hsp70, 70-kDa heat shock protein; ICER, inducible cAMP early repressor; LH-R, LH receptor; PGS-2, prostaglandin endoperoxidase synthase-2; PMSG, pregnant mare serum gonadotropin; StAR, steroidogenic acute regulatory protein.

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(ATFs) (23, 24), cAMP response element (CRE)-binding protein (CREB) (25–27), CRE modulatory protein (28–30), and CCAAT/enhancer binding proteins (C/EBPs) (31, 32). In animal cells, cAMP exerts many of its effects by activating cAMP-dependent protein kinase (2, 8, 33, 34). The free catalytic subunit of cAMP-dependent protein kinase phosphorylates CREB on serine 133 (26, 35). Phosphorylated CREB dimers bind to an atypical CRE in the inhibin α-subunit promoter, which, when mutated, no longer supports transcriptional activity of the promoter (36).

In addition to CREB, we have identified an isoform of the CRE modulatory protein family, the inducible cAMP early repressor (ICER), as a key regulator of inhibin gene transcription. ICER acts as a potent repressor of the inhibin α-subunit gene in ovarian granulosa cells (37). ICER is capable of repressing both basal as well as forskolin-stimulated levels of inhibin gene expression by competing with CREB homodimers for occupancy of the inhibin CRE located at −117 (38). ICER mRNAs are induced in granulosa cells of preovulatory follicles in response to human chorionic gonadotropin (hCG) or LH, and ICER proteins are induced in the ovary in response to gonadotropins (37). ICER expression in the ovary after the preovulatory LH surge is quite transient, because ICER is rapidly down-regulated within a few hours after hCG or LH administration (37). However, down-regulation of the inhibin α-subunit gene is maintained throughout the postovulatory period in the rodent ovary, extending beyond the period of ICER expression. Therefore, the transient nature of ICER expression in the ovary suggests that other transcription factors are necessary to maintain suppression of the inhibin α-subunit gene during ovulation and luteinization.

C/EBPβ is a member of the larger C/EBP family of transcription factors, which also includes the related proteins C/EBPα, C/EBPδ, CRP1/C/EBPε, and C/EBPγ. All C/EBP proteins are bZIP DNA-binding proteins with strong homology in their C-terminal region, and they recognize similar DNA motifs, such as the palindromic consensus C/EBP-binding site. C/EBP proteins are capable of dimerization with each other (39, 40); with other bZIP proteins, such as Fos or Jun (41), ATF4 (42), C/ATF (43), and CREB (44); and with other non-bZIP transcription factors, such as Rel domain proteins (45–48). C/EBP proteins have been shown to control the transcription of many important genes and can function as both activators and repressors of gene transcription (49–52).

The importance of C/EBPβ in female reproduction was clearly established with the finding that female mice deficient in C/EBPβ are sterile and exhibit periovulatory defects (53). Ovaries from adult female mutant mice are completely devoid of corpora lutea. Furthermore, ovaries from superovulated mutant mice show severely reduced numbers of ovulations and the presence of hemorrhagic antral follicles. At the molecular level, the lack of LH-responsive down-regulation of prostaglandin endoperoxidase synthase-2 (PGS-2) and aromatase transcripts in C/EBPβ-deficient ovaries compared with wild-type ovaries suggests that C/EBPβ is important in attenuating the expression of these genes in response to LH. We therefore tested the hypothesis that C/EBPβ is important in attenuating the expression of the inhibin α-subunit gene in response to LH. In these studies we provide evidence that C/EBPβ is expressed in the ovary after the LH surge and that it represses inhibin gene expression. We describe two distinct molecular mechanisms by which C/EBPβ exerts these effects. Our findings demonstrate that C/EBPβ plays a critical role in repression of the inhibin α-subunit gene in the ovary during the postovulatory period.

Materials and Methods

Animals and hormone treatments

C/EBPβ (−/−) mice were generated on a 129S1-C57BL/6 mixed strain background (53). Mice were kept on a 12-h light, 12-h dark cycle, with lights on at 0700 h. Mice were injected with pregnant mare serum gonadotropin (PMSG; 5 IU, ip; Sigma-Aldrich Corp., St. Louis, MO) for up to 48 h and with hCG (5 IU, ip; Sigma-Aldrich Corp.) for up to 7 h. All procedures were conducted in compliance with the guidelines of the Animal Care and Use Committee of the NCI.

Immature 22- to 24-d-old female Sprague Dawley rats (Charles River, Lexington, MA) were kept on 14-h light, 10-h dark cycles, with lights on at 0500 h. Rats were injected with PMSG (10 IU, sc; Sigma-Aldrich Corp.) for up to 48 h and subsequently with hCG (25 IU, ip; Sigma-Aldrich Corp.) for up to 144 h. Rats were killed at various time points, and ovaries were removed and either used immediately to isolate granulosa cells or rapidly frozen on dry ice and stored at −80 C. All animal protocols were approved by the animal care and use committee of Northwestern University (Evanston, IL), and animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

In situ hybridization

Ovarian sections from C/EBPβ-deficient and wild-type mice were processed for *in situ* hybridization as described previously (54), with a few modifications. An [^{35}S]UTP-labeled antisense riboprobe derived from rat inhibin α mRNA was used for hybridization. Prefixed paraffin sections were incubated with xylenes to remove paraffin and hydrated through an ethanol series before proceeding with prehybridization and hybridization procedures as described previously (54). Sense riboprobes were used as controls. Slides were then processed for emulsion autoradiography (NTB-2, Eastman Kodak Co., Rochester, NY). Exposure time on emulsion was 2 wk. After development of the slides, they were stained with hematoxylin to visualize nuclei.

RNA isolation and analysis

RNA from ovaries was isolated by homogenization in 4 M guanidium isothiocyanate containing 25 mM sodium citrate, 0.5% sarkosyl, and 7 μM β-mercaptoethanol and extraction with acid-phenol (55). Total ovarian RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase in the presence of deoxynucleosidyltriphosphates (1 mM) and random hexameric oligonucleotides, and aliquots of this cDNA were amplified by PCR with incorporation of [^{32}P]deoxy-CTP into the PCR product as described previously (54). PCR primers specific for the inhibin α-subunit were used: 5′-CTG GCC AAA GTG AAG GCA CTA and 3′-CAG GAA AGG AGT GGT CTC AGG (56). PCR primers specific for ribosomal protein L-19 were used as an internal control: 5′-CTG AAG GTC AAA GGG AAT GTG and 3′-GGA CAG AGT CTT GAT GAT CTC (57). The PCR products were size-separated on 5% polyacrylamide gels using electrophoresis. Gels were then dried, and PCR products were visualized by autoradiography on X-OMAT-AR film (Eastman Kodak Co.) and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

HeLaT4 and GRMO2 cell culture

HeLaT4 cells were cultured in DMEM supplemented with 5% fetal bovine serum in a humidified incubator at 37 C and 5% CO_2. GRMO2 cells (provided by N.V. Innogenetics, Ghent, Belgium) were cultured as previously described (58, 59) in HDTIS [1:1 mixture of DMEM and Ham's F-12 medium (Invitrogen Life Technologies, Inc., Gaithersburg, MD), 10 μg/ml insulin, 5 nM sodium selenite, and 5 μg/ml transferrin]

supplemented with 2% fetal bovine serum and sodium pyruvate (100 mg/liter) in a humidified incubator at 37 C and 5% CO_2.

Transfection and luciferase assays

GRMO2 cells were transiently transfected with DNA (60) using cationic liposomes prepared as described previously (61). Reporter plasmid DNA (500 ng) and C/EBPβ expression constructs (50 ng) were incubated at room temperature with lipofection reagent for 20 min in OptiMEM for each well of a 12-well culture dish. Cells were washed with PBS and incubated with the DNA-lipid mixture for 6 h. After 6 h, cells were aspirated and maintained in fresh HDTIS containing 2% fetal bovine serum for 14–16 h. Fresh medium or medium containing 10 μM forskolin was then added to the cells for 4–6 h. After forskolin treatment, cells were washed twice with PBS, and lysates were prepared for luciferase assays.

Transfected cells were lysed on ice in lysis buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 1 mM dithiothreitol (DTT), and 0.1% Triton X-100] for 20 min with gentle rocking. Luciferase assays were performed as previously described (62). Cell lysates (100 μl) were added to 400 μl reaction buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 5 mM ATP, 1 μg/ml BSA, and 1 mM DTT], 100 μl 1 mM luciferin (sodium salt; Analytical Bioluminescence, San Diego, CA) were added using an automatic injector, and emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence) for 10 sec. Relative light units were normalized for total protein content. Protein assays using the Bio-Rad Laboratories, Inc. (Richmond, CA), protein assay reagent were performed using 5 μl cell lysates.

Vaccinia virus expression of recombinant proteins

The vaccinia T7 RNA polymerase hybrid expression system (63) was used to overexpress proteins used in EMSAs. HeLaT4 cells were infected with vaccinia virus vTF7.3 expressing the bacteriophage T7 RNA polymerase (obtained under license from Dr. Bernard Moss, NIH, Bethesda, MD) at a multiplicity of infection of 10 for 35 min in PBS/0.1% BSA. Virus was aspirated, and preincubated DNA-liposome mixture was added to the cells and incubated for 6 h. Cells were washed once with PBS, maintained in culture for an additional 14–16 h, washed with PBS, harvested, and processed for protein isolation.

Granulosa cell culture

Ovaries from hormone-treated female rats were processed for follicular puncture as described previously (36, 64). Ovaries were collected into serum-free medium (4F) consisting of 15 mM HEPES (pH 7.4), 50% DMEM, and 50% Ham's F-12 with transferrin (5 μg/ml), human insulin (2 μg/ml), hydrocortisone (40 ng/ml), and antibiotics. Ovaries were washed once in 4F, incubated at 37 C for 30 min in 4F medium containing 0.5 M sucrose and 10 mM EGTA, and washed once in fresh 4F medium. Using a 25-gauge needle under a dissection microscope, individual follicles were punctured, and the granulosa cells were extruded. Nuclear protein extracts were then prepared from these cells.

Preparation of whole ovary, whole cell, and nuclear protein extracts

Protein lysates were prepared in lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 1 mg/ml antipain, aprotinin, leupeptin, pepstatin, and 1 mM NaF]. Frozen tissue samples were pulverized in dry ice using a mortar and pestle and collected in lysis buffer. Samples were homogenized with six to eight strokes of a glass-glass Dounce homogenizer (Kontes Co., Vineland, NJ). This homogenate was subjected to two cycles of freezing and thawing. The lysates were centrifuged to remove nuclear debris, and the supernatant was collected and frozen at −80 C.

Small-scale nuclear extracts were prepared as previously described (65). Cultured cells were washed twice with cold PBS, scraped from the plates in cold PBS with protease inhibitors, and collected in microfuge tubes. Cells were spun at low speed, and PBS was aspirated. The cells were gently resuspended in 5 packed cell vol buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, and 1 mg/ml each of antipain, aprotinin, leupeptin, and pepstatin] and incubated on ice for 10 min. Lysates were then homogenized on ice using 10 strokes of a glass-glass Dounce homogenizer. The homogenate was centrifuged at 1500 × *g* for 2 min to collect the nuclear pellet, which was then resuspended in two thirds packed volume of buffer C [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% (vol/vol) glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, and 1 mg/ml each of antipain, aprotinin, leupeptin, and pepstatin]. Protein concentrations were estimated using a Bradford colorimetric assay (Bio-Rad Laboratories, Inc.).

Western blot analysis

Protein lysates were boiled for 5 min in sample buffer and size-separated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose (BA-85, Schleicher & Schuell, Keene NH). The membrane was washed once with water and blocked with 3% nonfat dry milk in PBS (blocking buffer) for 20 min at room temperature with shaking. The membrane was incubated with primary antibody, anti-C/EBPβ at 1:4000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-Flag at 1:6000 (Sigma), in blocking buffer for 16–18 h at 4 C with gentle shaking, then washed twice with water, followed by a 90-min incubation at room temperature with goat antirabbit antibody conjugated to horseradish peroxidase (1:5000; Promega Corp., Madison, WI) or sheep antimouse antibody conjugated to horseradish peroxidase (1:5000; Promega Corp.). The membrane was washed with water twice, with PBS/0.05% Tween for 5 min, and with water four or five times. The antibody-antigen complexes were visualized using an enhanced chemiluminescence system (ECL-Plus kit, Amersham Biosciences, Little Chalfont, UK).

Primary and secondary antibodies were removed by incubating the membrane in 62 mM Tris (pH 6.8), 100 mM β-mercaptoethanol, and 2% sodium dodecyl sulfate for 30 min at 50 C. The membrane was washed twice with PBS for 10 min, reblocked for 20 min at room temperature with shaking, incubated with primary antibody, anti-α-inhibin (66, 67), at 1:1000 (W. Vale and J. Vaughan, The Salk Institute, La Jolla, CA) in blocking buffer for 48 h at 4 C with gentle shaking, and then washed twice with water, followed by a 90-min incubation at room temperature with goat antirabbit antibody conjugated to horseradish peroxidase (1:5000; Promega Corp.). Subsequent washes and enhanced chemiluminescence detection were identical to those described previously.

The membrane was stripped a second time and incubated with primary antibody, antiactin, at 1:500 (Sigma) in blocking buffer for 48 h at 4 C with gentle shaking, then washed twice with water, followed by a 90-min incubation at room temperature with goat antirabbit antibody conjugated to horseradish peroxidase (1:5000; Promega Corp.). Subsequent washes and enhanced chemiluminescence detection were identical to those described previously.

EMSAs

Double-stranded nucleotide probes (160 bp) spanning the inhibin α-subunit promoter were generated by PCR using [^{32}P]deoxy-CTP. The following primers were used to generate these probes: −769rIna5′-CGG GCC CGA GCC CAG AAC, −617rIna3′-GGG GAA AGA GGC CCA GAG GC, −628rIna5′-CCT CTT TCC CCT CCT CCC TC, −453rIna3′-CCT GCT CTA TCT ATA CTT AGC C, −463rIna5′-ATA GAG CAG GCA GGA CCA CCT C, −301rIna3′-AGG GTT GGC CAG TCA TCA AAT C, −311rIna5′-TGG CCA ACC CTA AGC ACC CTG, −144rIna3′-TAT CTC CCA CTC CCG CCA AGA ATG, −154rIna5′-AGT GGG AGA TAA GGC TCA GGG C, and +2rIna3′-TTC CCT CCC CAT CCC ACT GCT TG. Double-stranded oligonucleotide probes (36 bp) that span the C/EBPβ nonconsensus site and inhibin nonconsensus CRE were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega Corp.) and gel-purified on a 12% polyacrylamide gel. Nuclear lysates (2–30 μg) from vaccinia-infected transfected cells or primary granulosa cell cultures were used for each binding reaction. Lysates were incubated in binding buffer [10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, and 200 ng/μl poly deoxy(inosinic-cytidylic)acid] for 20 min at room temperature with 10,000 cpm ^{32}P-labeled probe. Where indicated, 3 μl anti-C/EBPβ or anti-CREB (Santa Cruz Biotechnology, Inc.) antibody were added to the reaction before labeled probe, and the reaction was incubated on ice for 30–60 min. Also where indicated, 20× unlabeled competitor oligonucleotide or normal rabbit serum (Animal Pharm Services,

Inc., San Francisco, CA) was added to the binding reaction before the addition of probe. The protein-DNA complexes were resolved on a native 5% polyacrylamide-Tris-borate-EDTA gel. The gel was dried and exposed to x-ray film (Kodak XAR).

Statistical analysis

An unpaired two-tailed *t* test was performed using statistical analysis functions in PRISM 3.0 (GraphPad, Inc., San Diego, CA). Differences between the activities of the indicated constructs were considered statistically significant at $P < 0.05$.

Results

Misregulation of inhibin α-subunit mRNA in C/EBPβ-null ovaries

To determine whether inhibin α-subunit mRNA levels were altered in ovaries from C/EBPβ-null mice, ovarian sections from wild-type and C/EBPβ-null mice injected with exogenous gonadotropins were analyzed by *in situ* hybridization using a [^{35}S]UTP-labeled inhibin α antisense riboprobe corresponding to the full-length inhibin α-subunit. Two wild-type mice and two knockout mice were injected with PMSG for 48 h and subsequently with hCG for 7 h. One ovary from each animal was used in the *in situ* hybridization, and the other ovary was used in later RT-PCR studies. As shown in Fig. 1A, in ovaries from wild-type mice injected with PMSG for 48 h and hCG for 7 h, there was almost a complete absence of detectable α-subunit expression in preovulatory follicles (marked by *white arrows*), which is expected based on previous work (68, 69). In contrast, ovaries from similarly treated C/EBPβ-null mice still had subsets of preovulatory follicles (marked by *white arrows*) that continued to express detectable levels of inhibin mRNA in granulosa cells (Fig. 1A).

To quantify these differences, the second ovary from each animal was used to prepare RNAs that were examined for inhibin α-subunit mRNA expression using an RT-PCR assay, as shown in Fig. 1B. After 7 h of hCG stimulation, C/EBPβ mutant ovaries demonstrated higher levels of gene expression than similarly treated wild-type ovaries. Quantification of RT-PCR results showed a 3-fold elevation of inhibin α mRNA levels in C/EBPβ-null ovaries compared with wild-type ovaries. These data suggest that the lack of C/EBPβ disrupts the down-regulation of the α-subunit gene in ovarian granulosa cells that is normally seen in wild-type mice.

Regulation of C/EBPβ protein expression in ovary

To identify the temporal expression and regulation of C/EBPβ isoforms in rodent ovary, protein lysates were prepared from ovaries harvested from hormone-treated immature rats. Rats were treated with PMSG for various times or with PMSG for 48 h, followed by hCG for various times. Western blotting was performed using an anti-C/EBPβ antibody that detects all known isoforms of C/EBPβ (Fig. 2A), anti-α inhibin (66, 67) (Fig. 2B), and antiactin to control for total protein levels (Fig. 2C). As shown in Fig. 2A, in the absence of hormonal stimulation, none of the C/EBPβ isoforms was detected in the ovary. After 10 h of PMSG stimulation, three C/EBPβ isoforms, corresponding to molecular masses of 36, 34, and 14 kDa, were expressed at low levels. Expression of these isoforms almost completely disappeared by 48 h post PMSG stimulation. In response to subsequent hCG stimulation, C/EBPβ isoforms were again induced, but to higher levels than those observed after PMSG stimulation alone. Expression was maximal 12 h after hCG treatment and remained elevated 24 h post hCG treatment. Although expression of all isoforms was down-regulated by 48 h post hCG treatment, detectible expression continued until 6 d after hCG treatment. In Fig. 2B, the expression of α inhibin precursor protein increased after PMSG stimulation, with maximal expression 48 h post-PMSG treatment. The expres-

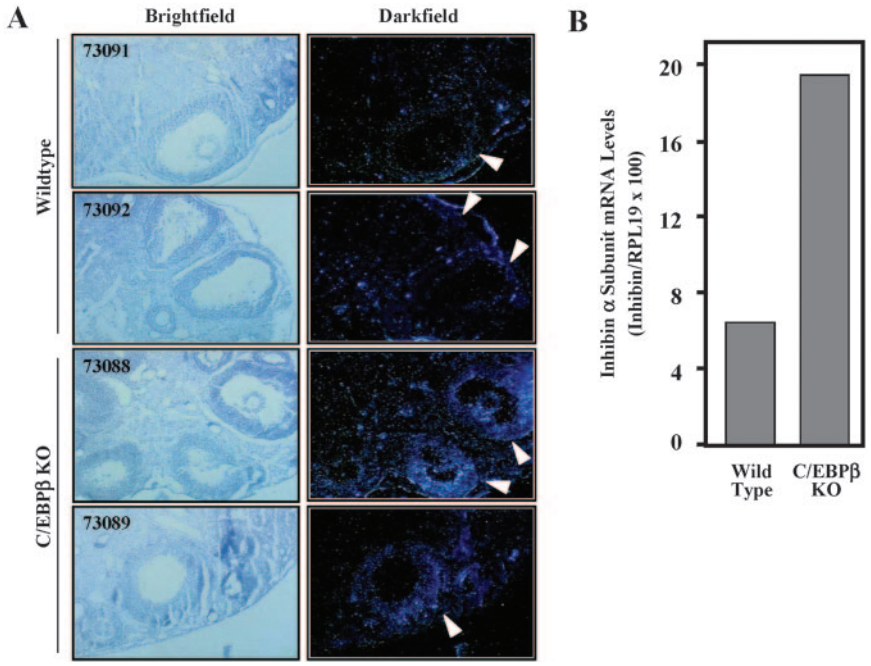

FIG. 1. Effects of C/EBPβ deficiency on inhibin α-subunit mRNA expression in the mouse ovary. A, The *panels within columns* include bright- and dark-field photographs of *in situ* hybridization using a rodent 5′-inhibin α antisense cRNA probe at ×100 magnification. The *panels within rows* include ovarian photographs from wild-type or C/EBPβ knockout mice. The panels are ovarian sections from immature female mice treated with PMSG for 48 h, followed by hCG for 7 h. Two wild-type mice and two knockout mice were injected, and one ovary from each animal was examined (n = 2 ovaries). *Arrows* point to follicles in the ovary. B, The second ovary from each group used in the *in situ* hybridization was also collected (n = 2 ovaries), and RNAs isolated from these ovaries were analyzed by RT-PCR for inhibin α mRNAs. All RNA samples were also analyzed by RT-PCR for ribosomal protein L19 mRNA as an internal control. Results were quantified using a PhosphorImager, and the relative levels of inhibin α-subunit mRNAs normalized to the L19 mRNA control are plotted.

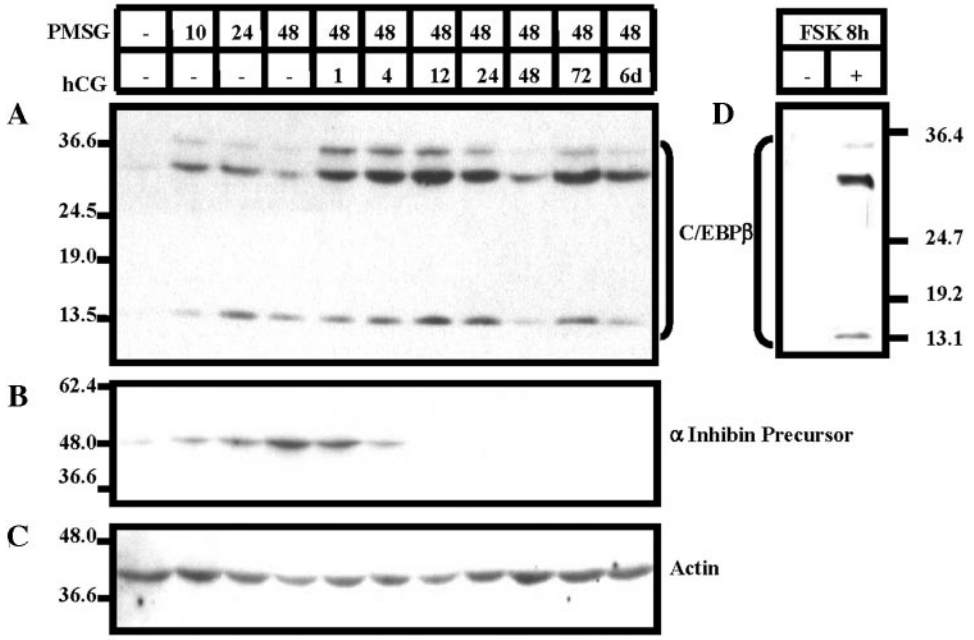

FIG. 2. Expression of C/EBPβ protein in the rat ovary. Protein lysates (200 μg) extracted from the ovaries of immature rats treated with hormones as indicated were separated by electrophoresis using a 10% SDS-PAGE gel. Proteins were electrophoretically transferred to a nitrocellulose membrane and immunostained using an anti-C/EBPβ antibody that detects all C/EBPβ isoforms, followed by a goat antirabbit antibody conjugated to horseradish peroxidase (A). Enhanced chemiluminescence was used to detect protein complexes. The same protein blot shown in A was washed and stripped to remove primary and secondary antibodies and incubated with an antiinhibin α antibody (B) or an antiactin antibody (C) and developed as described in A. D, Similar Western blot analysis using nuclear extracts (40 μg) from GRMO2 cells treated with 10 μM forskolin (FSK) for 8 h or untreated. The positions of C/EBPβ isoforms, α inhibin precursor, and actin are indicated, along with the positions of molecular mass size standards.

sion of α inhibin precursor protein diminished in response to subsequent hCG stimulation and was not detected by 12 h after hCG treatment. This pattern of inhibin α protein expression followed the pattern of inhibin α-subunit mRNA expression in the ovary (37). When GRMO2 cells (58, 59), a mouse granulosa cell line, are treated with forskolin, a pharmacological agent that activates adenylyl cyclase and induces intracellular cAMP, C/EBPβ protein was induced (Fig. 2D), strongly suggesting that the induced expression of C/EBPβ occurs within granulosa cells of the ovary.

Inhibin α-subunit gene repression by C/EBPβ in granulosa cells

To determine whether C/EBPβ could functionally repress the inhibin α-subunit gene, transient transfections were performed in GRMO2 cells. A C/EBPβ expression construct coding for the 34-kDa isoform of C/EBPβ was cotransfected with a 769-bp inhibin α-subunit promoter-luciferase reporter construct, and basal and forskolin-induced luciferase activity were measured. Figure 3A shows the luciferase activity nor-

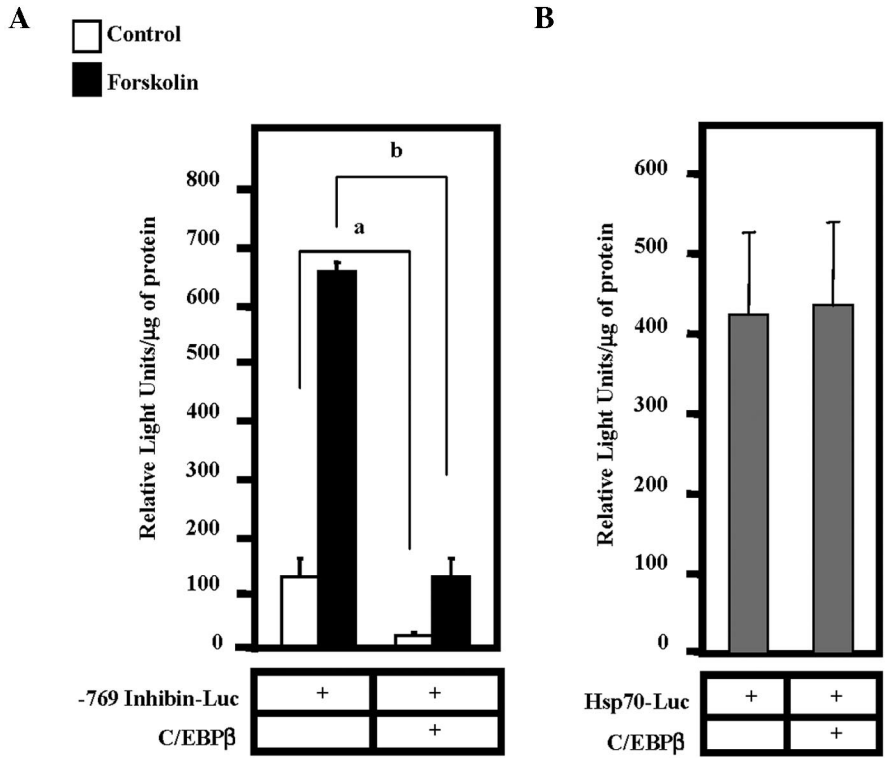

FIG. 3. Effects of C/EBPβ overexpression on inhibin α-subunit promoter activity in GRMO2 cells. Luciferase activity in GRMO2 cells transiently cotransfected with the indicated DNAs. A, Cells were transfected for 6 h, allowed to recover 16 h, treated with 10 μM forskolin (■) or vehicle (□) for 6 h (A) or left untreated (▨; B), and then harvested for luciferase activity. Values are relative light units per microgram of cellular protein and represent the mean ± SEM (n = 3). These results represent multiple independent experiments (n > 4 independent transfections). Reference levels for both panels are the same. Both basal and forskolin activity levels of the −769 inhibin-Luc construct without C/EBPβ were statistically significant compared with the respective values obtained for the −769 inhibin-Luc construct with C/EBPβ: a, $P < 0.05$; b, $P < 0.0001$. Activity levels of the Hsp70-Luc construct with or without C/EBPβ were not statistically significant.

malized for total protein. The inhibin α-subunit promoter demonstrated a basal level of activity in GRMO2 cells that was induced 6-fold by 10 μM forskolin treatment. Cotransfection with the C/EBPβ expression construct significantly repressed both basal and forskolin-induced promoter activity, although the fold forskolin response with C/EBPβ overexpression remained about 6-fold. When a control 70-kDa heat shock protein (Hsp70) promoter was cotransfected with C/EBPβ, as shown in Fig. 3B, promoter activity was not altered, indicating that the repressive effect of C/EBPβ on the inhibin α promoter was specific.

Definition of C/EBPβ-binding sites in the inhibin α promoter

To examine whether C/EBPβ could directly interact with the inhibin α-subunit gene promoter, EMSAs were performed. HeLaT4 cells were transfected with the C/EBPβ expression construct using a vaccinia virus T7 RNA polymerase-based system (63) to overexpress recombinant protein. Five radiolabeled, double-stranded, nucleic acid probes approximately 160 bp in length and spanning the 800-bp inhibin α proximal promoter region were generated by PCR. As shown in Fig. 4A, a major DNA-protein complex was formed when using the probe spanning the −628 to −453 region of the promoter. Additional, less intense DNA-protein complexes were seen with probes spanning other regions of the promoter, and Fig. 4B is a longer autoradiographic exposure of a subsequent EMSA spanning the −154 to +2 region, showing the presence of a C/EBPβ-specific DNA-protein complex. Although C/EBPβ can bind to multiple regions of the promoter, these EMSAs demonstrate that the major binding site is located within the −628 to −453 region. This region contains a nonconsensus site at −522 to −513 previously shown to bind C/EBPβ in the steroidogenic acute regulatory (StAR) gene promoter (70). To determine whether C/EBPβ was binding specifically to this site, a 36-bp radiolabeled double-stranded oligonucleotide probe flanking this nonconsensus binding site was designed, and the EMSA was repeated. Figure 5 shows that a DNA-protein complex is formed when the probe is incubated with lysates containing C/EBPβ (lane 3). The mobility of this DNA-protein complex was further reduced when the reaction was incubated with a C/EBPβ antibody (lane 6) and was unaffected when a nonspecific antibody was included in the reaction (lane 9). Finally, when 20-fold excess cold competitor DNA was included in the reaction (lane 12), no DNA-protein complex was observed. When four nucleotides were mutated in this binding site (lane 15), the DNA-protein complex was substantially reduced, indicating that the mutation diminished binding of C/EBPβ to this site in the promoter.

To determine whether endogenous C/EBPβ protein binds to the nonconsensus C/EBPβ-binding site at −522 to −513,

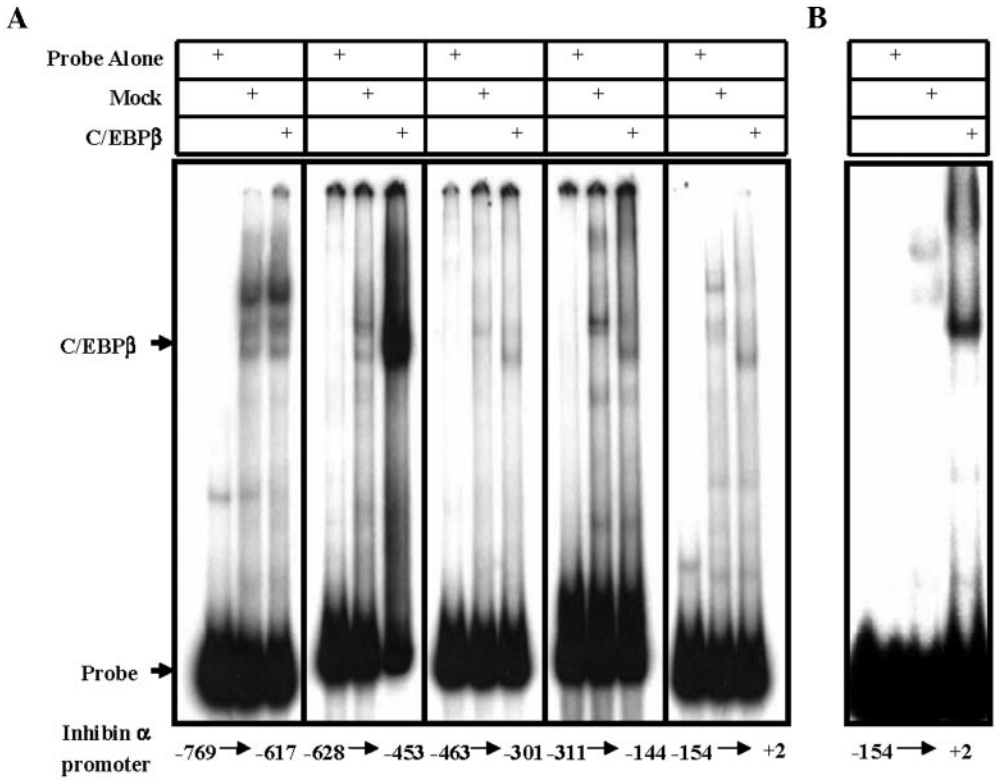

FIG. 4. Binding of C/EBPβ to the inhibin α-subunit promoter. Nuclear extracts (2 μg) from vaccinia T7-infected HeLaT4 cells transfected with a C/EBPβ expression construct (C/EBPβ) or empty vector (Mock) were incubated with oligonucleotide probes generated by PCR with incorporation of [^{32}P]deoxy-CTP into the PCR product. Probe, without an extract (Probe Alone) was also included on the gel. A, Five probes spanning the −769 to +2 inhibin α-subunit promoter were generated, and the regions are indicated below the gel. DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. The specific protein complex is indicated by the *arrow* labeled C/EBPβ, and the free probe is indicated by the *arro*w labeled Probe. B, Longer exposure of another gel shift with the probe spanning the −154 to +2 inhibin α-subunit promoter.

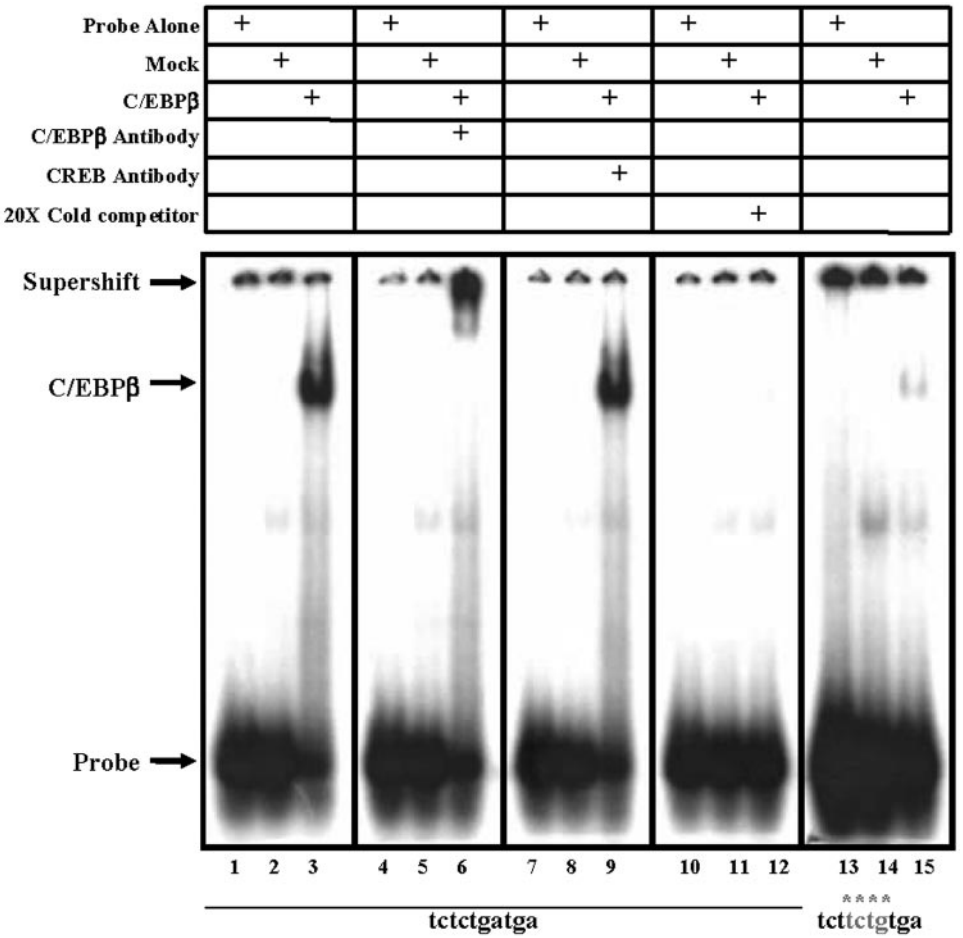

FIG. 5. Binding of C/EBPβ to a nonconsensus site in the inhibin α-subunit promoter. Nuclear extracts (2 μg) from vaccinia T7-infected HeLaT4 cells transfected with a C/EBPβ expression clone (C/EBPβ) or empty vector (Mock) were incubated with a ^{32}P-labeled double-stranded oligonucleotide probe that spans the nonconsensus C/EBPβ-binding site (lanes 1–12) or a mutant nonconsensus C/EBPβ-binding site (lanes 13–15). The sequences for these sites are *below* the gel, and the mutated nucleotides are indicated (****). Probe without an extract (Probe Alone) was also included on the gel. The binding reaction was incubated with an anti-C/EBPβ antibody (C/EBPβ antibody), an anti-CREB antibody (CREB antibody), or a 20-fold excess of nonconsensus oligo (×20 cold competitor). DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. The protein complex corresponding to C/EBPβ is indicated by the *arrow* labeled C/EBPβ, the antibody-supershifted complex is indicated by the *arrow* labeled Supershift, and the free probe is indicated by the *arrow* labeled Probe.

nuclear extracts from granulosa cells isolated from rats treated with PMSG alone or with PMSG and subsequently hCG for different times were incubated with the 36-bp radiolabeled, double-stranded oligonucleotide probe used in Fig. 5. Figure 6 shows binding of C/EBPβ to this nonconsensus binding site after 4 and 12 h of hCG stimulation (lanes 5 and 8), which was diminished when a C/EBPβ antibody was incubated in the reaction (lanes 6 and 9). Incubation with normal rabbit serum did not diminish the shifts (lanes 7 and 10). Extracts from PMSG alone-treated animals did not demonstrate binding of C/EBPβ, consistent with the data in Fig. 2 showing that C/EBPβ was not abundantly expressed at this time.

To determine how a mutation in the C/EBPβ-binding element would affect transcription from the α-subunit gene promoter, the mutation described in Fig. 5 was introduced into the −769 bp inhibin promoter-luciferase reporter, and this construct was transiently transfected into GRMO2 cells. There was significant enhancement of both basal and forskolin-stimulated activities of the mutant promoter compared with the wild-type promoter (Fig. 7). This finding is consistent with endogenous C/EBPβ acting in a repressive fashion at this site to control inhibin gene transcription. However, when a C/EBPβ expression construct was cotransfected with the mutant promoter construct, both basal and forskolin-stimulated levels were repressed, although the repressed levels were similar to those of the wild-type promoter in the absence of C/EBPβ and were still elevated compared with those of the wild-type promoter. Thus, when the major binding site for C/EBPβ in the inhibin α-subunit promoter was mutated, C/EBPβ could still exert a partial repressive effect, suggesting a second mechanism by which C/EBPβ can regulate the inhibin α-subunit gene promoter.

C/EBPβ inhibits binding of CREB to the inhibin α-subunit CRE

Although mutation of the C/EBPβ-binding site increased basal activity of the inhibin promoter 2-fold, incorporation of a second mutation at the CRE element at −117 (which alone dramatically reduced promoter activity) abolished this increase in basal promoter activity (data not shown). This observation suggests that C/EBPβ actions may be dependent in part on the proximal promoter CRE. To test this idea, a shorter inhibin promoter construct containing the CRE, but lacking the −522 C/EBPβ-binding site, was examined. This −146 bp promoter construct demonstrated an overall decrease in promoter activity compared with the −769 bp construct, similar to other inhibin promoter deletion constructs reported previously (36). Although overall promoter activity of the −146 bp construct was diminished, it was still subject to C/EBPβ repression (Fig. 8), indicating that C/EBPβ can have a secondary effect on this proximal promoter region. To determine whether C/EBPβ might inhibit binding of CREB

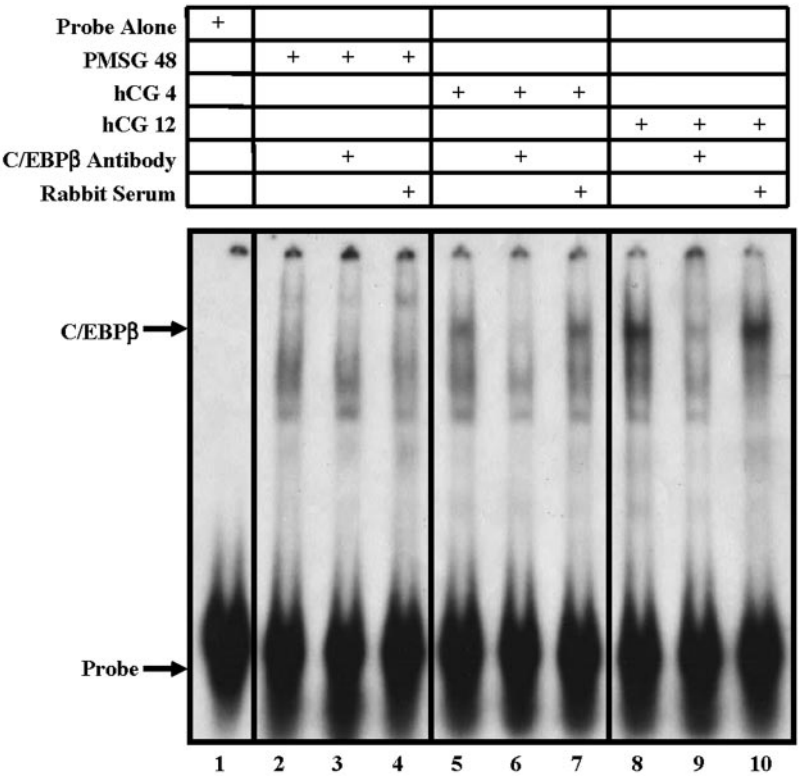

FIG. 6. Binding of endogenous C/EBPβ in granulosa cells to the nonconsensus C/EBPβ site in the inhibin α-subunit promoter. Nuclear extracts (30 μg) from granulosa cells isolated from rats treated with PMSG or with PMSG and subsequently with hCG for the indicated times were incubated with a ^{32}P-labeled, double-stranded oligonucleotide probe that spans the nonconsensus C/EBPβ binding site. Probe without an extract (Probe Alone) was also included on the gel. The binding reaction was incubated with an anti-C/EBPβ antibody (C/EBPβ antibody) or with normal rabbit serum (rabbit serum). DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. The protein complex corresponding to C/EBPβ is indicated by the *arrow* labeled C/EBPβ, and the free probe is indicated by the *arrow* labeled Probe.

to the CRE, HeLaT4 cells were cotransfected with constant amounts of CREB DNA and increasing amounts of C/EBPβ DNA, and vice versa, and nuclear lysates were prepared. Western blot analysis of these protein lysates was performed to control for protein amounts. Cotransfection of C/EBPβ DNA did not diminish the expression of CREB protein and vice versa, shown in Fig. 9A. An EMSA was performed using a probe containing the nonconsensus CRE present in the inhibin α promoter. As shown in Fig. 9B, when CREB was transfected in the absence of C/EBPβ, a CREB protein-DNA complex was seen (lane 3). When C/EBPβ was transfected in the absence of CREB, a C/EBPβ protein-DNA complex was also seen (lane 7), indicating that the weak binding to this region observed in Fig. 4 represents binding to the CRE. Antibodies against both CREB and C/EBPβ supershifted each respective protein-DNA complex (data not shown). EMSAs with a labeled α-inhibin mutant CRE did not show binding of either CREB or C/EBPβ (lane 10), verifying that C/EBPβ was indeed binding to the CRE. Increasing the amount of C/EBPβ cotransfected with CREB resulted in a decrease in the amount of CREB binding, demonstrating that C/EBPβ inhibited the binding of CREB to the inhibin α promoter (Fig. 9C). However, increasing the amount of CREB cotransfected with C/EBPβ did not alter the amount of C/EBPβ binding, demonstrating that CREB cannot inhibit the binding of C/EBPβ to the inhibin α promoter. These data are quantified in Fig. 9C.

Discussion

Maintaining normal cyclicity in female rodents requires the complex coordination and regulation of a multitude of hormones and signaling mechanisms. Positive and negative feedback loops within the reproductive axis control the stimulation and suppression of important regulatory hormones that, in turn, regulate ovarian-specific genes crucial to the timed growth and development of ovarian follicles, ovulation, and the subsequent formation of the corpus luteum. One ovarian gene, the inhibin α-subunit gene, is induced by basal FSH and down-regulated by the preovulatory LH surge. This down-regulation of the inhibin α-subunit gene during late proestrus leads to a decrease in dimeric inhibin (68), relieving suppression of FSH and allowing for a secondary FSH surge on the morning of estrus (69). In response to the secondary FSH surge, a new cohort of follicles is recruited for growth, leading to a renewal of the follicular cycle (71). The down-regulation of the inhibin α-subunit gene, and subsequently dimeric inhibin, is a crucial step in the maintenance of the estrous cycle, as evidenced by the altered ovulation in transgenic mice constitutively expressing α inhibin (72). Although the inhibin α-subunit gene is used as a model system for understanding how ovarian signal transduction mechanisms and gene expression are mediated by the gonadotropins, aspects of these studies can probably be related to other ovarian genes that are coordinately regulated with inhibin α, such as the aromatase, FSH-R, LH-R, estrogen receptor β, protein kinase A regulatory subunit RIIβ, inhibin β_A, and inhibin β_B genes.

In this study the mechanisms by which C/EBPβ represses the inhibin α-subunit gene were examined. Our initial interest in this bZIP transcription factor arose when C/EBPβ-deficient mice exhibited severe reproductive and ovulatory defects. The idea that C/EBPβ might function in regulation of the inhibin α-subunit gene initiated from the finding that ovaries from C/EBPβ-deficient mice fail to down-regulate

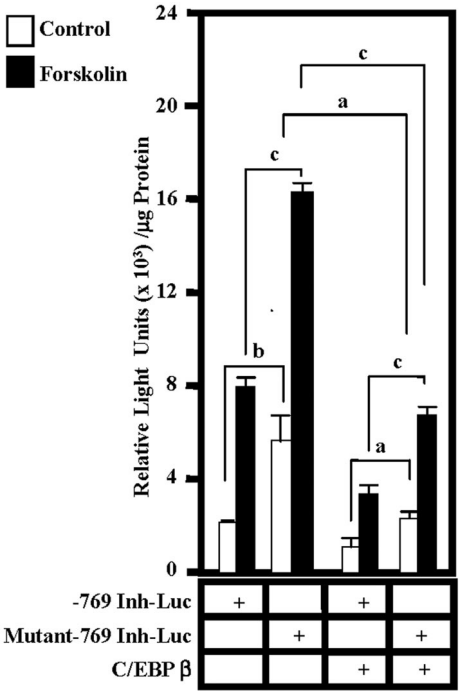

FIG. 7. Effects of a mutation in the nonconsensus C/EBPβ-binding site on inhibin α-subunit promoter activity. GRMO2 cells were transiently transfected with the indicated DNAs. Mutant −769 inhibin-luciferase contains the four-nucleotide mutation shown in Fig. 5. Cells were transfected for 6 h, allowed to recover 16 h, treated with 10 μM forskolin (■) or vehicle (□) for 6 h, and then harvested for luciferase activity. Values are relative light units per microgram of protein and represent the mean ± SEM (n = 3). These results represent multiple independent experiments (n > 3 independent transfections). Activities obtained from mutated promoter constructs were statistically significant compared with the respective values obtained for the wild-type inhibin-luciferase construct in the absence or presence of C/EBPβ: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.0001$.

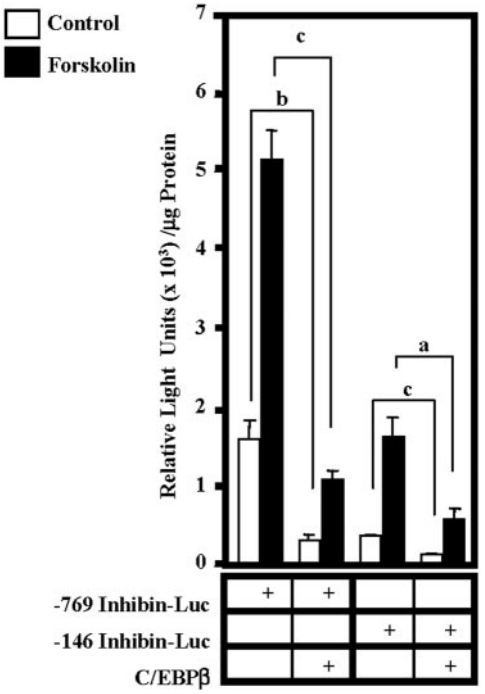

FIG. 8. Effects of C/EBPβ on the transcriptional activity of inhibin α-subunit promoter deletion constructs. GRMO2 cells were transiently transfected with the indicated DNAs. Cells were transfected for 6 h, allowed to recover for 16 h, treated with 10 μM forskolin (■) or vehicle (□) for 6 h, and then harvested for luciferase activity. Values are relative light units per microgram of protein and represent the mean ± SEM (n = 3). These results represent multiple independent experiments (n > 3 independent transfections). Activities of each promoter cotransfected with C/EBPβ were statistically significant compared with the respective values obtained for each promoter in the absence of C/EBPβ: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.0001$.

both the aromatase and PGS-2 genes in response to hCG (53). Because the inhibin α-subunit gene is regulated in a manner similar to aromatase and is down-regulated in response to LH/hCG, we investigate whether this gene would fail to be down-regulated in these C/EBPβ-deficient ovaries after PMSG/hCG stimulation. Our results indicate that in C/EBPβ-deficient ovaries, at the same time that the aromatase and PGS-2 genes are misregulated, inhibin mRNA levels are also misregulated. These findings are consistent with the hypothesis that C/EBPβ is necessary to repress the inhibin α-subunit gene after the LH surge.

C/EBPβ expression has been shown to be both FSH and LH/hCG responsive (70, 73). To confirm this expression pattern of C/EBPβ protein in the immature rat ovarian model as well as in the GRMO2 cell culture model and to verify which isoforms were present, Western protein blot analysis was performed. C/EBPβ is expressed in the rodent ovary in response to gonadotropin stimulation and in granulosa cells in response to forskolin stimulation. The predominant translational isoform of C/EBPβ in both the ovary and granulosa cells is the 34-kDa isoform. Low levels of C/EBPβ are induced by PMSG treatment, but are down-regulated to near basal amounts by 48 h after PMSG treatment. Subsequent treatment with hCG causes a robust increase in protein amounts, which continues throughout the hCG treatment regimen. This pattern of C/EBPβ expression is consistent with C/EBPβ acting as a repressor of the inhibin α-subunit gene during the periovulatory period.

There are four reported isoforms of C/EBPβ ranging in molecular mass from 14–38 kDa. The most abundant isoforms are the 34-kDa isoform (74, 75) and the 20-kDa isoform. These different isoforms can be generated by translation from downstream in-frame initiation codons (74, 76–78) as well as proteolytic cleavage (79, 80). In our expression studies, shorter C/EBPβ species in cells transfected with a full-length C/EBPβ cDNA are not observed; however, a 14-kDa form is observed in ovarian and granulosa cell lysates that appears to be identical to the 14-kDa proteolytic product (79).

Only one other C/EBP family member, C/EBPα, has shown regulated expression in the ovary. The expression pattern for C/EBPα is quite different from that for the C/EBPβ isoforms. C/EBPα mRNA is induced by about 2-fold in immature rat ovaries within 24 h of PMSG injection, and this expression is maintained 48 h after gonadotropin treatment (81). Subsequent treatment with hCG results in a down-regulation of C/EBPα mRNA to basal levels. It is proposed that C/EBPα is involved in follicular growth and maturation before ovulation (81).

C/EBPβ proteins have been implicated in both positive and negative regulation of gene expression in a variety of

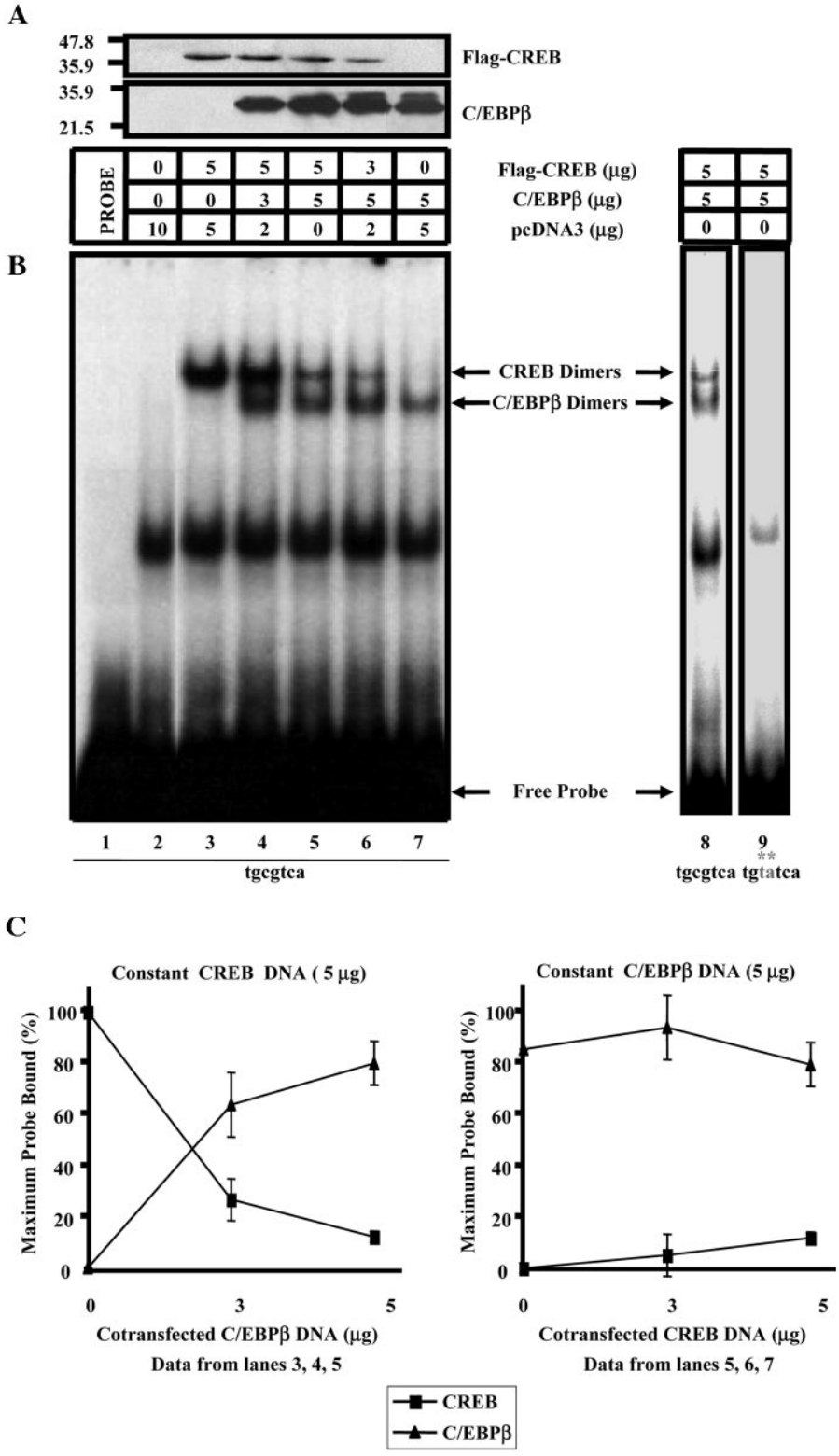

FIG. 9. Competition of C/EBPβ with CREB for binding to the inhibin α subunit CRE. A, Nuclear extracts (10 μg) from vaccinia T7-infected HeLaT4 cells transfected with constant amounts of CREB DNA and increasing amounts of C/EBPβ, and vice versa, were processed for Western blotting to control for protein expression. pcDNA3 is the parent vector for both Flag-CREB and C/EBPβ expression constructs. Proteins were separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The *top portion* of the blot (35.9–47.8 kDa) was immunostained using a Flag antibody, followed by a sheep antimouse antibody conjugated to horseradish peroxidase. The lower portion of the blot (21.5–35.9 kDa) was immunostained using a C/EBPβ antibody, followed by a goat antirabbit antibody conjugated to horseradish peroxidase. The detection system used was enhanced chemiluminescence. The positions of molecular mass size standards are indicated. B, Nuclear extracts (2 μg) were incubated with a ^{32}P-labeled, double-stranded oligonucleotide probe that spans either the nonconsensus CRE in the inhibin α-subunit promoter (lanes 1–8) or a mutant CRE (lane 9). The sequences for these sites are *below* the gel, and the mutant nucleotides are indicated (**). DNA-protein complexes were electrophoresed on a 5% native polyacrylamide gel and visualized by autoradiography. The protein complexes corresponding to CREB and C/EBPβ dimers are indicated by correspondingly labeled *arrows*. Free probe is indicated by the *arrow* labeled Probe. C, Probe bound to the different protein complexes was quantified from four independent experiments and the content of probe, presented as the maximum probe bound (percentage) ± SEM (n = 4), was plotted against the amount of C/EBPβ DNA cotransfected with CREB DNA.

tissues. Full-length proteins contain transactivation domains as well as DNA binding and dimerization domains at the C-terminal (82). Shorter isoforms lack the transactivation domain, yet still contain the bZIP motif, thereby retaining dimerization and DNA-binding abilities (74, 83). C/EBPβ isoforms can exert their actions on various genes by binding to gene promoters to regulate transcription. In addition to a consensus C/EBPβ-binding motif, there are numerous nonconsensus binding sites. C/EBPβ has been shown to bind to consensus binding sites in ovarian specific gene promot-

ers, such as P450 aromatase (84) and PGS-2 (73), and to a nonconsensus binding site in the StAR promoter (70). Binding of full-length C/EBPβ to the P450 aromatase promoter diminishes promoter activity (84), whereas binding of full-length C/EBPβ to the StAR promoter increases promoter activity (70), thereby demonstrating C/EBPβ's divergent actions on different gene promoters.

Experiments described here identify a nonconsensus C/EBPβ binding site positioned at −522 to −513 in the inhibin α-subunit gene promoter. Recombinant C/EBPβ protein as well as endogenous C/EBPβ protein from ovarian granulosa cells isolated from rats treated with PMSG and subsequently hCG exhibit binding to this site. Therefore, at times when C/EBPβ is expressed in the rodent ovary (post hCG stimulation), C/EBPβ can bind to the nonconsensus C/EBPβ-binding site in the inhibin α-subunit gene promoter. Interestingly, the nonconsensus C/EBPβ-binding site identified here is identical to the one that binds C/EBPβ to the promoter of the StAR gene (70) and is necessary to activate StAR gene transcription. In that study, a specific antiserum to C/EBPα supershifted the C/EBPβ complex, suggesting that a C/EBPα/C/EBPβ heterodimer was involved in activation of this gene. This is consistent with other studies demonstrating the importance of the dimerization partner for C/EBPβ. Due to the down-regulation of C/EBPα during the periovulatory period, it is not likely that C/EBPβ is heterodimerizing with C/EBPα to regulate the inhibin α-subunit promoter.

Transfection studies investigating the functional importance of this upstream site demonstrated elevated levels of transcriptional activity when the site is mutated. Elevated activity with the mutant promoter is consistent with endogenous C/EBPβ acting as a repressor at this site. The ability of C/EBPβ to exert partial repressive effects in the presence of this mutation, however, implied that C/EBPβ could act elsewhere on the promoter. Because C/EBPβ belongs to the bZIP family of transcription factors, one hypothesis was that C/EBPβ could heterodimerize with another protein to exert repressive effects on the α-subunit promoter. Heterodimerization between CREB and C/EBPβ has been shown in one other system (44); therefore, it is possible that C/EBPβ forms inactive heterodimers with CREB. The competitive gel-shift experiment with cotransfected CREB and C/EBPβ looked at the interactions of both proteins with each other as well as the inhibin CRE. In this experiment, only bands that correspond to CREB dimers and C/EBPβ dimers are observed. Heterodimers between the two in the absence of DNA do not readily form in an *in vitro* assay (data not shown). In cotransfected cells at intermediate levels of both CREB and C/EBPβ, competition for the CRE is seen. Binding of C/EBPβ to the CRE is weak compared with that to the −522 site, as demonstrated in the EMSAs presented in Fig. 4. Consistent with this difference in binding affinity *in vitro*, EMSA studies looking at binding of endogenous C/EBPβ induced in response to hCG in granulosa cell extracts to the inhibin α-subunit gene promoter demonstrated binding to the upstream nonconsensus C/EBPβ site (Fig. 6), but not to the CRE (data not shown). This suggests that although C/EBPβ can demonstrate partial repressive effects on the proximal promoter of the α-subunit gene, the predominant mechanism of repression of the inhibin α-subunit gene by C/EBPβ *in vivo* is probably through binding to the −522 binding site.

The ability of C/EBP proteins to function as activators and repressors of gene transcription in a variety of tissues clearly demonstrates the importance of this family of transcription factors in a number of cellular processes. Our studies demonstrate that the full-length, 34-kDa isoform of C/EBPβ is capable of acting as a repressor in this model. Based on these current findings, as well as our previous studies investigating the role of ICER as a repressor in this system (37, 38), we can propose a model for how inhibin α-subunit gene expression is regulated during the postovulatory period in the rodent ovary. On the afternoon of proestrus, before the LH surge, inhibin levels are maximal. Immediately after the LH surge, ICER is induced in the ovary and rapidly represses target genes such as α-inhibin. Within a few hours, ICER has already begun to disappear from the ovary. At the same time, C/EBPβ is induced, begins repressing target genes such as α-inhibin, and maintains this repression throughout the postovulatory period. It is clear that signaling by the gonadotropins and cAMP involves a number of regulatory factors, and these studies of the role of C/EBPβ in this process contribute to our understanding of the signaling events involved in regulation of cAMP-responsive, ovarian-specific genes.

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Address all correspondence and requests for reprints to: Dr. Kelly E. Mayo, Department of Biochemistry, Molecular Biology, and Cell Biology, and Center for Reproductive Science, Northwestern University, Evanston, Illinois 60208. E-mail: k-mayo@northwestern.edu.

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