

Gonadotropins Regulate Inducible Cyclic Adenosine 3',5'-Monophosphate Early Repressor in the Rat Ovary: Implications for Inhibin α Subunit Gene Expression

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Many hormones that stimulate intracellular signaling pathways utilizing the second messenger cAMP affect gene expression in target cells through the activation of cAMP-responsive transcriptional regulatory proteins. Two of the best characterized of these are the cAMP-response element (CRE)-binding protein (CREB) and the CRE-modulatory protein (CREM). CREB and CREM are expressed as a family of proteins that have diverse activities in either stimulating or repressing gene transcription. In this study we examined the expression and regulation of the CREM gene in the rat ovary and in granulosa cells, to determine whether repressor isoforms of CREM might have a role in the LH-mediated suppression of inhibin α -subunit gene expression that occurs just before ovulation. We found that the predominant CREM mRNAs in the ovary correspond to previously described internal transcripts of the CREM gene that encode the inducible cAMP early repressor (ICER). ICER mRNAs are strongly induced in the ovary by exogenous gonadotropins in immature rats and are transiently expressed in the ovary immediately after the preovulatory LH surge in adult cycling rats. Although ICER is expressed in multiple ovarian cell types, expression in granulosa cells is observed only in response to LH stimulation. ICER mRNAs are also induced by the activation of

cAMP-signaling pathways in cultured primary granulosa cells. To determine whether ICER can act as a functional repressor to modulate potential target genes such as the inhibin α -subunit gene, an ICER expression construct was transiently co-transfected into a granulosa cell line along with an inhibin α -subunit promoter-luciferase reporter gene. Both basal and cAMP-induced expression of the inhibin α -subunit promoter were suppressed by ICER. These studies reveal that CREM, a tissue-specific factor, is expressed and regulated by gonadotropins in the ovary, that the predominant CREM transcripts encode the repressor protein ICER, and that ICER is capable of inhibiting cAMP-induced expression of the inhibin α -subunit gene. Our findings are consistent with a role for repressors such as ICER in mediating the suppression of inhibin α -subunit gene expression that occurs in the ovary at the time of the preovulatory LH surge. (Molecular Endocrinology 12: 785-800, 1998)

INTRODUCTION

During the rodent estrous cycle, FSH secreted from the pituitary stimulates the growth and maturation of ovarian follicles, and this is accompanied by increased expression of many FSH-responsive genes within the granulosa cells of the growing follicle. One such target gene encodes the α -subunit of inhibin, a dimeric gonadal hormone that, in turn, acts to suppress the syn-

thesis and secretion of pituitary FSH (1). Another FSH target gene is that encoding the LH receptor (2, 3), and once the mature ovarian follicle acquires functional LH receptors, the preovulatory LH surge on proestrus afternoon triggers the morphological and biochemical changes associated with ovulation of the oocyte and luteinization of the remaining follicle cells. In response to the LH surge, the inhibin α -subunit gene is rapidly down-regulated, leading to decreased inhibin secretion and providing an environment permissive to the prolonged elevation (the secondary surge) of FSH secretion that occurs on estrus morning and is critical for the recruitment of a new cohort of ovarian follicles (for reviews see Refs. 1, 4, and 5).

This basic pattern of FSH stimulation and LH repression during the estrous cycle is common to a number of genes expressed in the ovary, including aromatase (6), the LH receptor (2), the FSH receptor (7), and the inhibin α - and β -subunits (8). Other genes are similarly induced by FSH in ovarian granulosa cells, but their expression remains elevated after the LH surge and during the process of luteinization (9). Thus, determining the molecular mechanisms through which gonadotropins exert these effects on gene expression is important for understanding the normal control of the ovarian cycle and promises to lend insight into disruptions of this control that might be associated with reproductive dysfunction, such as infertility.

The gonadotropins FSH and LH act through stimulatory G protein-coupled receptors expressed on target cells (10, 11) and transduce their signal, at least in part, by the activation of adenylyl cyclase and the production of the second messenger cAMP (12, 13). Recent findings suggest that the gonadotropins can also act through signaling pathways that lead to the activation of protein kinase C (14, 15) or intracellular tyrosine kinases (16). While it is not known how FSH and LH might differentially regulate gene expression, the number of LH receptors far exceeds the number of FSH receptors on the granulosa cells of preovulatory follicles (17), and the levels of intracellular cAMP generated by FSH in small antral follicles are much lower than those generated by LH in preovulatory follicles (18–20), suggesting that the magnitude of the gonadotropin-induced cAMP signal might regulate the resultant transcriptional responses.

Many of the transcriptional responses to cAMP are thought to be mediated by a family of cAMP-responsive factors that belong to the larger superfamily of bZip proteins, so named for the basic region and leucine zipper motifs that form the DNA binding and dimerization domains of these proteins (21–23). Perhaps the best characterized of these factors are the cAMP-response element (CRE)-binding protein (CREB) (24–26) and the CRE-modulatory protein (CREM) (27–29). These proteins have a very similar domain structure, and both CREB and CREM are expressed as a family of isoforms that can act as cAMP-regulated stimulators or repressors of gene transcription (26, 27, 30–32). CREB is thought to be fairly

ubiquitously expressed, whereas CREM expression is tissue-specific and is highly regulated in neuroendocrine tissues and cell types (27).

Alternative RNA processing leads to the formation of mRNAs that encode diverse isoforms of the CREB and CREM proteins. The full-length forms of these proteins, which include a kinase-inducible (phosphorylation) domain and one or more glutamine-rich domains with transactivation function, act as transcriptional activators. For example, CREB 341 and CREB 327 are strong activators of transcription (33, 34), and CREM τ can augment CREB-mediated activation of cAMP-responsive genes (30). Shorter isoforms of these proteins that include the DNA-binding and dimerization domains but lack the kinase-inducible or transactivation domains can act as transcriptional repressors. The proteins I-CREB (35), CREM α , β , γ , and S-CREM (27, 32) are examples of such repressor isoforms. These repressor proteins might act either by occupying CRE sites in target gene promoters as transcriptionally inactive homodimers or by forming inactive heterodimers with CREB or other transcriptional activators (32, 36–38). One of the best characterized of these repressor isoforms, and the focus of this study, is the inducible cAMP early repressor (ICER) (39, 40). The ICER mRNA is transcribed from an intronic promoter of the CREM gene and encodes a protein that includes DNA-binding and dimerization domains but lacks the kinase-inducible and transactivation domains, and thus acts as a potent repressor. The intronic promoter that regulates ICER expression is itself cAMP responsive, and ICER therefore autoregulates its expression. ICER has been proposed to be a key signal that is transiently induced to attenuate cAMP-dependent signaling pathways (39).

We reported previously that the full-length activator isoform of CREB is expressed in the rat ovary and that the pituitary gonadotropins do not regulate CREB gene expression but rather stimulate CREB phosphorylation, leading to its activation (41). We also demonstrated that CREB is a key mediator of FSH-stimulated inhibin α -subunit gene expression in the ovarian granulosa cell (42). Given the increasing evidence for modulatory effects of CREM isoforms on CREB-mediated gene transcription, we wanted to test the hypothesis that CREM isoforms play a role in attenuating the transcriptional response of the inhibin α -subunit gene to gonadotropins and cAMP in ovarian granulosa cells. In the present study we investigate the expression and regulation of CREM/ICER mRNA and protein in immature rats treated with exogenous gonadotropins, in adult rats during the estrous cycle, and in cultured rat granulosa cells treated with hormones *in vitro*. We also test directly the effect of ICER overexpression on inhibin α -subunit gene promoter activity in a granulosa cell line. Our findings are consistent with an important role for CREM/ICER in mediating the repression of the inhibin α -subunit gene that is observed in the ovary immediately after the preovulatory LH surge.

RESULTS

Gonadotropin Regulation of CREM/ICER mRNAs in the Rat Ovary

To determine whether the CREM gene is expressed in the ovary and is regulated by gonadotropin-induced cAMP signaling pathways, immature rats were treated with exogenous gonadotropins, and ovarian sections were analyzed by *in situ* hybridization using a [³⁵S]UTP-labeled CREM antisense riboprobe. This probe corresponds to the full-length τ 2 form of CREM, as shown schematically in Fig. 1. Figure 2 indicates that CREM mRNA is induced in the ovary within 4 h of PMSG treatment and returns to basal levels by 48 h after PMSG treatment. In PMSG-primed animals, CREM mRNA is strongly induced within 1 h of human CG (hCG) treatment and again returns to basal levels by 12 h after hCG treatment.

To establish whether the CREM transcripts detected in the ovary correspond to the full-length forms of CREM or the shorter repressor forms such as ICER, specific probes were designed to detect these two mRNA species. A 47-nucleotide antisense oligonucle-

otide specific to the unique intronic sequences found in the ICER mRNA was used to detect ICER, while a CREM τ 2 5'-probe corresponding to the kinase-inducible and glutamine-rich domains was used to detect full-length forms of CREM (all probes are indicated in Fig. 1). As shown in Fig. 3, the ICER-specific mRNA expression pattern in the hormonally treated rat ovary corresponded closely to that observed previously using the full-length CREM probe. In contrast, transcripts hybridizing to the 5'-CREM probe were expressed at very low levels and were not induced by gonadotropins in the immature rat ovary (Fig. 3). Thus, inducible CREM expression in the ovary appears to represent a selective activation of the internal P2 promoter leading to the generation of ICER mRNAs.

While the gonadotropin-treated immature rat is an excellent and easily manipulated model for mimicking events that occur during the normal reproductive cycle in mature animals, we wanted to assess whether ICER would also be induced in cycling female rats by the normal preovulatory LH surge. RNA blot analysis was therefore used to detect ICER transcripts in ovaries isolated from rats at different times during the 4-day estrous cycle. Serum FSH and LH levels were simultaneously determined in these animals. As shown in Fig. 4A, ICER transcripts of 1700 and 1900 nucleotides were observed only at 1800 and 2000 h on proestrus. These times correspond well to the preovulatory LH surge, which began at 1600 h and peaked at 2000 h on proestrus (Fig. 4B). Treatment of the animals with either pentobarbital or a GnRH antagonist (WY-45760), which block the preovulatory LH surge (43–45), also abolished ICER mRNA induction at 1800 h proestrus (Fig. 4C). These data indicate that, in the ovary, ICER is rapidly induced and then down-regulated in response to the LH surge in animals progressing through a normal reproductive cycle.

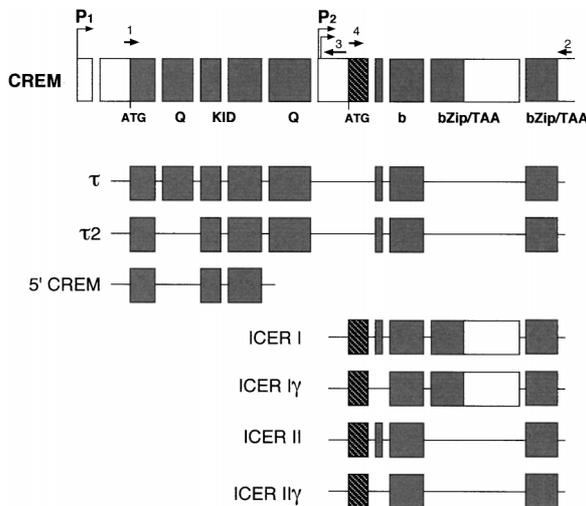


Fig. 1. CREM and ICER cDNA Clones and Oligonucleotide Primers

A schematic diagram of the CREM protein is shown at the top. The boxes represent exons of the CREM gene. The shaded boxes are coding regions, and the cross-hatched box corresponds to a region unique to the ICER isoforms. The two alternative promoters, P1 and P2, and the alternative initiation codons (ATG) used to generate full-length CREM isoforms vs. ICER are indicated. Glutamine-rich domains (Q), the kinase-inducible domain (KID), and two basic-leucine zipper domains (bZip), each followed by a stop codon (TAA), are shown. The numbered arrows represent the positions and orientations of synthetic oligonucleotide primers used in this study. Below the CREM structure are schematic diagrams of the cDNA clones or subclones used in this study, including CREM τ and τ 2 testis cDNAs, a 5'-CREM subclone prepared from the τ 2 cDNA, and cDNAs encoding the four ovarian ICER isoforms.

Cell-Specific Expression of ICER in the Ovary

Although ICER is induced in the immature rat ovary in response to both PMSG and hCG, we found that the cellular localization of ICER transcripts was quite distinct after induction by these two gonadotropins. As shown in Fig. 5, ICER mRNA is localized predominantly to thecal and interstitial cells after PMSG stimulation. In contrast, after hCG stimulation, ICER mRNA is also localized to the granulosa cells of most large preovulatory follicles. Interestingly, in all cases, ICER mRNA was found to colocalize with LH receptor mRNA (Fig. 5). This suggests that the induction of ICER by PMSG in thecal and interstitial cells may be mediated by the weak LH activity of PMSG as opposed to its predominant FSH-like properties (46).

To investigate further this cell type-specific induction of ICER, the granulosa cell and thecal-interstitial cell compartments were separated from ovaries of animals treated with exogenous gonadotropins and used to prepare RNAs that were examined for ICER mRNA expression using an RT-PCR assay. The PCR

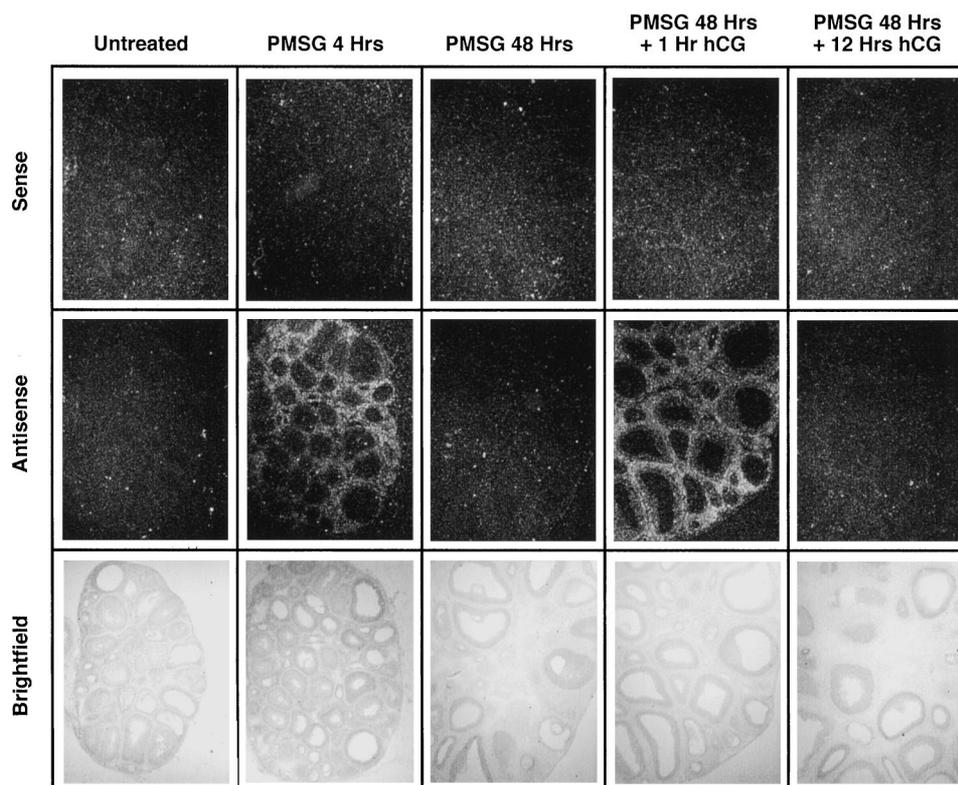


Fig. 2. Expression and Regulation of CREM mRNA in the Immature Rat Ovary

The panels within rows include darkfield photomicrographs of *in situ* hybridization using a CREM sense cRNA probe (top row), darkfield photomicrographs of *in situ* hybridization using a CREM antisense cRNA probe (middle row), or brightfield photomicrographs of the corresponding ovarian sections (bottom row). The panels within columns include photographs of sections of ovaries from immature rats that were either untreated or treated with PMSG for 4 or 48 h and subsequently treated with hCG for 1 or 12 h.

primers used in this assay are indicated in Fig. 1. As shown in Fig. 6, ICER mRNA is induced in the thecal and interstitial cell-enriched fraction by both PMSG and hCG treatment, whereas ICER mRNA is induced in the granulosa cell-enriched fraction only after hCG treatment. Figure 6 also indicates that four predominant ICER transcripts were detected by RT-PCR. Each of these was cloned and sequenced, and this analysis indicated that they correspond to the four previously characterized forms of ICER, designated I, I γ , II, and II γ (39). The exon structures of these four ICER isoforms are schematically shown in Fig. 1.

Gonadotropin and cAMP Regulation of ICER mRNAs in Granulosa Cells

To establish whether ICER is expressed and regulated in granulosa cells maintained in primary culture, granulosa cells were isolated from the ovaries of immature rats that were either untreated (naive cells) or were treated with 10 IU of PMSG for 48 h (primed cells). The cells were treated with gonadotropins or the adenylyl cyclase activator forskolin for various times, RNA was isolated, and ICER mRNA expression was monitored using an RT-PCR assay. As shown in Fig. 7, all four of

the ICER transcripts described in the previous section were detected. These transcripts could be induced by a high dose of recombinant human FSH in the naive cells, by a lower dose of hCG in the primed cells, or by the nonspecific agent forskolin in both cell populations. In the PMSG-primed cells treated with hCG *in vitro*, ICER expression was highly elevated at 1 h, decreased substantially at 4 h, and returned to basal levels at 12 h after hormone addition (Fig. 7).

The ability of forskolin to mimic the induction of ICER by gonadotropins suggests that a cAMP-dependent pathway leading to the activation of protein kinase A (PKA) is involved. To confirm this, and to examine the ability of ICER to be induced in an immortalized mouse granulosa cell line that was used in a subsequent experiment, GRMO2 cells (47, 48) were treated with recombinant FSH or hCG for 1 h in the presence or absence of H89, an inhibitor of PKA (49). Figure 8 demonstrates that both recombinant FSH and hCG induce ICER mRNAs in these cells, and this induction is inhibited by greater than 90% by H89. Thus, ICER mRNAs are rapidly and transiently induced through a PKA-dependent mechanism in GRMO2 granulosa cells.

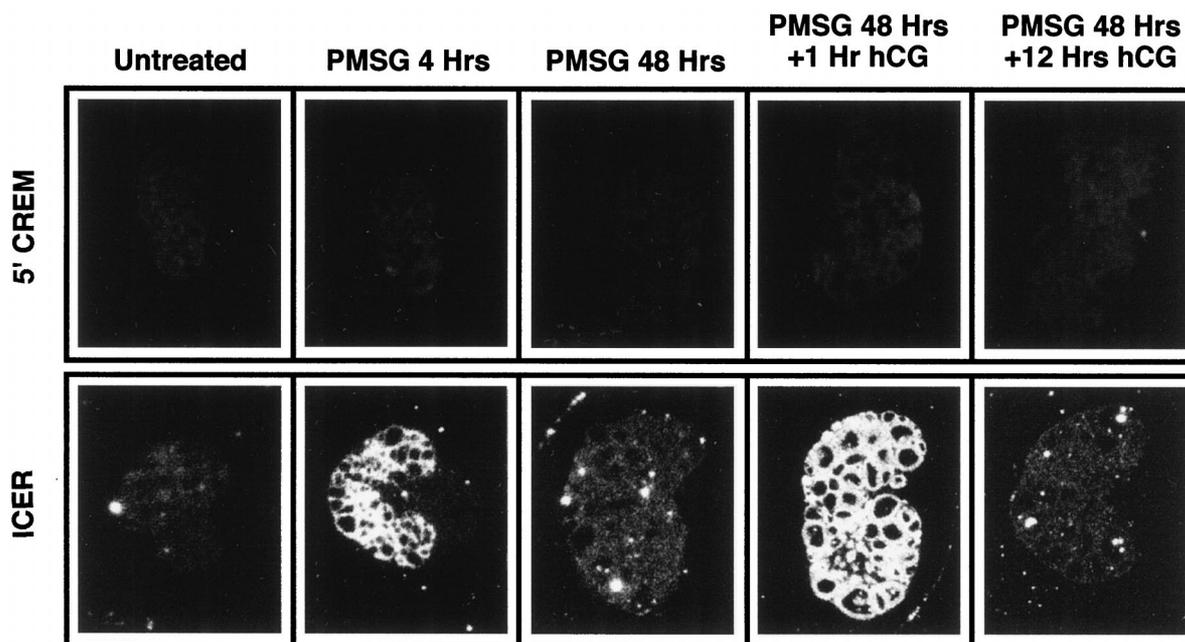


Fig. 3. Comparison of 5'-CREM and ICER mRNA Expression in the Rat Ovary

The panels within rows include darkfield photomicrographs of *in situ* hybridization using a 5'-CREM antisense cRNA probe (top row) or darkfield photomicrographs of *in situ* hybridization using an ICER antisense oligonucleotide probe (bottom row). The panels within columns include photographs of sections of ovaries from immature rats that were either untreated or treated with PMSG for 4 or 48 h and subsequently treated with hCG for 1 or 12 h.

ICER Expression and Inhibin α -Subunit Gene Repression

The temporal relationship between ICER induction and inhibin α -subunit gene repression after LH administration was examined using the immature rat model. RNAs prepared from granulosa cells that had been isolated from the ovaries of rats treated with PMSG for various times or treated with PMSG for 48 h followed by hCG for various times were used to assess ICER and inhibin α -subunit mRNA expression. As shown in Fig. 9, the levels of inhibin α -subunit mRNA increased in response to PMSG stimulation and peaked at 48 h of hormone treatment. After hCG administration, inhibin α -subunit mRNA was substantially decreased by 4 h and had returned to basal levels by 12 h of hormone treatment. In these same RNA samples, ICER mRNAs were not affected by PMSG treatment, were strongly induced within 1 h of hCG treatment, and thereafter were down-regulated. Thus, there is a tight temporal correlation between the onset of ICER expression and the hCG-induced suppression of inhibin α -subunit gene expression.

To extend these studies to examine the expression of ICER protein in the ovary, protein extracts were prepared from the granulosa cell and thecal-interstitial cell fractions of hormonally treated immature rats. Western protein blots were performed using an anti-CREM antibody that detects ICER (32). As shown in Fig. 10A, in the thecal-interstitial fraction three protein species ranging from 17–20 kDa were induced at low

levels within 4 h of PMSG treatment and declined to basal levels by 48 h after PMSG treatment. Subsequent hCG treatment rapidly induced ICER proteins in these cells. ICER protein expression peaked at 4 h after hCG treatment and was down-regulated by 12 h after hCG treatment. In the granulosa cell fraction, shown in Fig. 10B, ICER proteins were also induced at 4 h after hCG treatment and then down-regulated (PMSG alone had no effect on granulosa cell ICER protein expression, consistent with the mRNA results). These results indicate that ICER protein is maximally expressed at 4 h after hCG stimulation, corresponding closely to the time when inhibin α -subunit mRNA begins to decrease.

To establish whether the ICER protein can directly interact with the nonconsensus CRE element in the inhibin α -subunit promoter, electrophoretic mobility shift assays were performed. A radiolabeled double-stranded oligonucleotide probe corresponding to the inhibin α -subunit CRE was incubated with whole cell extracts from GRMO2 cells that were transiently transfected with an ICER I isoform expression construct using a vaccinia virus-T7 RNA polymerase-based system (50). As shown in Fig. 11, this extract produced a shift of the α -inhibin CRE probe that could be competed with an excess of the unlabeled α -inhibin CRE or a consensus CRE, but not with a nonspecific oligonucleotide. This band was not observed when extracts from nontransfected GRMO2 cells were used. When an antibody against the CREM protein (which detects ICER) was included in the incubation, a supershifted

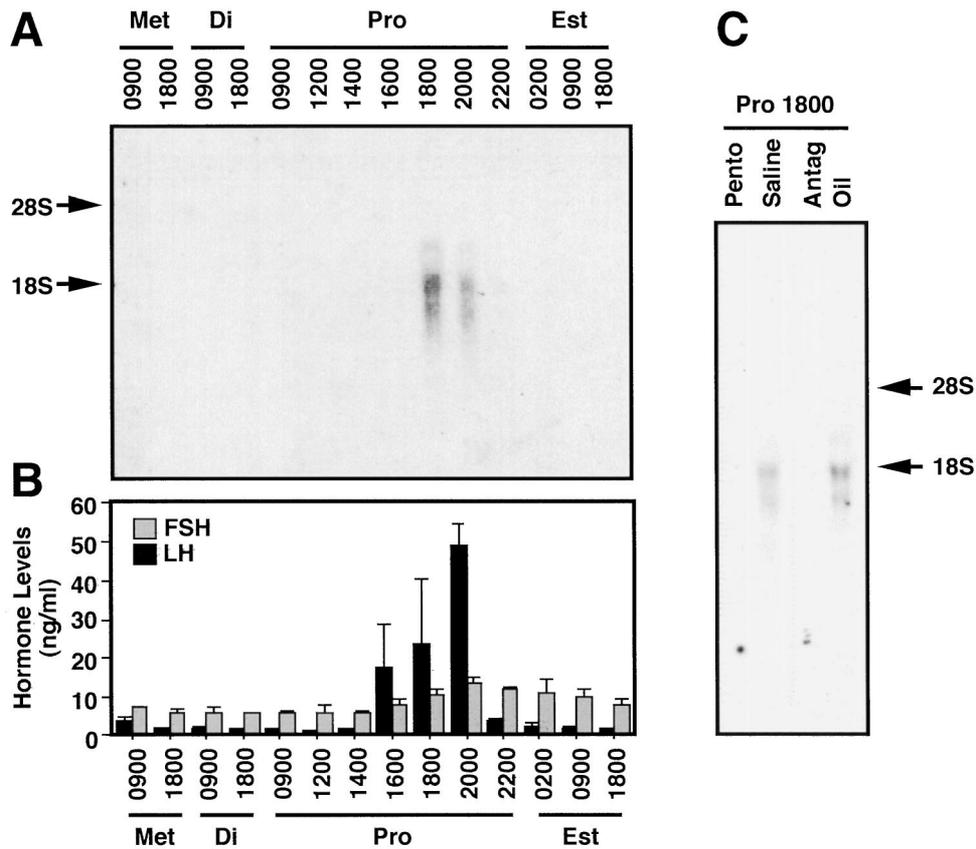


Fig. 4. Expression of ICER mRNA in the Rat Ovary during the Estrous Cycle

Panel A, RNAs (20 μ g) isolated from the ovaries of adult rats at the indicated times during the estrous cycle were examined by Northern RNA blotting using an ICER-specific oligonucleotide probe. The positions of the 18S and 28S ribosomal RNA markers are indicated. Panel B, Levels of FSH and LH in trunk blood from the same group of animals analyzed for ICER mRNA in panel A were determined by RIA. Error bars indicate the SEM, n = 3. Panel C, Northern RNA blot analysis of ICER expression in the ovaries of rats that were treated with saline or pentobarbitol (Pento) at 1330 h proestrus in one experiment and with oil or the GnRH antagonist WY-45760 (Antag) at 1200 h proestrus in another experiment. Ovaries were isolated at 1800 h proestrus in both experiments. RNA blots were subsequently hybridized with a ribosomal protein S2 probe to verify equivalent loading and transfer of all RNA samples (not shown).

complex of lesser mobility was observed, indicating that ICER is a component of the protein complex formed on the inhibin α -subunit CRE.

To determine directly if ICER could act as a functional repressor of the inhibin α -subunit gene, the same ICER I expression construct described above was used. This construct, or an antisense control, was cotransfected into the GRMO2 granulosa cell line along with an inhibin α -subunit promoter-luciferase reporter construct, and basal and forskolin-induced luciferase activity was measured. Figure 12A shows the structures of the two constructs used, while Fig. 12B shows the luciferase activities normalized for total protein. As expected, the inhibin α -subunit promoter was induced by forskolin in these cells, although the induction is modest at this early time after forskolin stimulation (4 h). The sense ICER construct substantially reduced both basal and forskolin-induced promoter activity, while the antisense ICER construct had essentially no effect. These studies indicate that

ICER-I can act as a functional repressor of basal and cAMP-stimulated inhibin α -subunit gene expression in granulosa cells.

DISCUSSION

Hormonal signals that are transduced through cAMP-dependent mechanisms in many tissues and cell types lead to the activation of cAMP-responsive transcription factors such as CREB, CREM, and the activating transcription factors (ATFs) (22, 51, 52). Tremendous diversity in the forms and actions of these factors is likely to provide for the fine tuning of cAMP-dependent transcriptional responses in such target cells. This diversity is generated at multiple levels, including numerous related genes encoding bZip factors (53), the use of alternative promoters and RNA processing to produce mRNAs encoding functionally distinct pro-

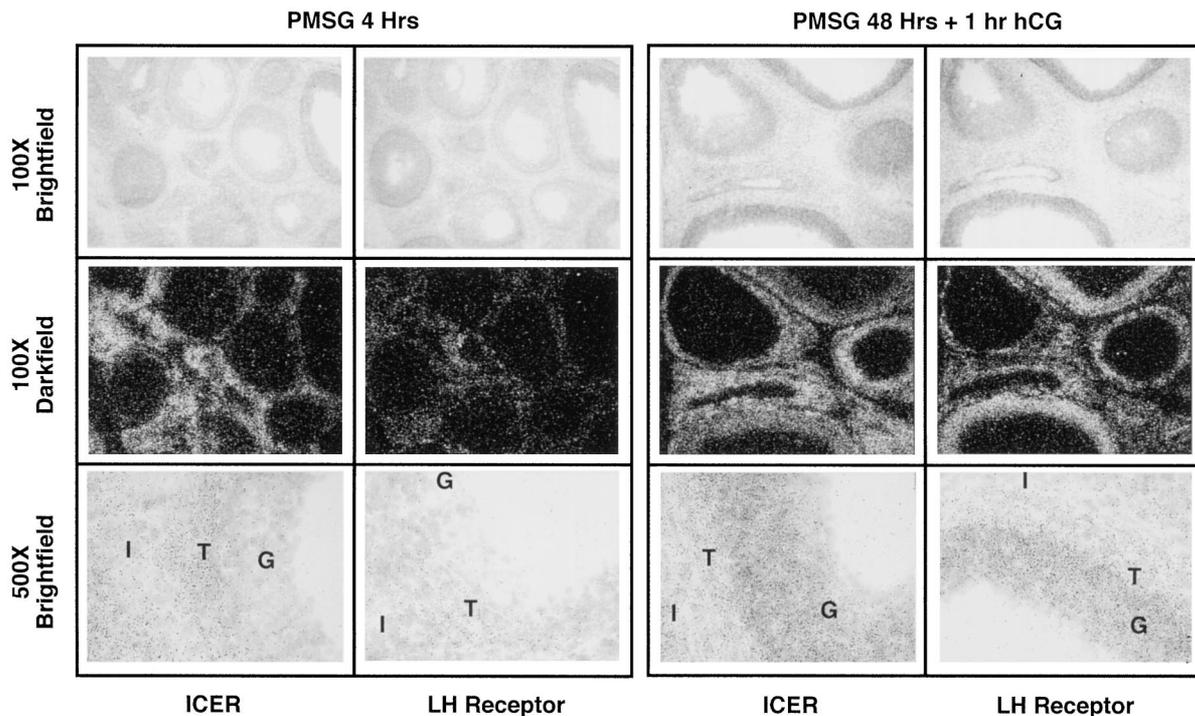


Fig. 5. Cell-Specific Expression of ICER mRNA in the Gonadotropin-Treated Rat Ovary

Brightfield and darkfield photomicrographs at 100 \times magnification and brightfield photomicrographs at 500 \times magnification of *in situ* hybridization using ICER or LH receptor antisense cRNA probes. The *left-hand* panels are ovarian sections from immature rats treated with PMSG for 4 h. The *right-hand* panels are ovarian sections from immature rats treated with PMSG for 48 h followed by hCG for 1 h. In the high magnification brightfield photographs, theca cells (T), interstitial cells (I), and granulosa cells (G) are indicated.

teins (26, 30, 39, 54), the posttranslational modification of these factors by phosphorylation (33, 55, 56), and the ability of these factors to dimerize and heterodimerize in a combinatorial fashion to generate specificity for distinct types of DNA response elements (38, 55, 57–60). Of particular interest for this study, unique products of the CREB and CREM genes can act either as cAMP-regulated activators or repressors of target gene transcription (30, 35, 39). Perhaps the best characterized of the repressor isoforms is the ICER, and in this report we describe the expression and regulation of ICER in the rat ovary and test the hypothesis that ICER is an important regulator of a model cAMP-responsive target gene, the inhibin α -subunit gene.

Unlike CREB, which is fairly ubiquitously expressed, CREM and its isoforms are expressed in a tissue- and cell-specific manner (27). In particular, CREM isoforms are often highly expressed and regulated in neuroendocrine tissues and cells, with well studied examples being the pineal gland (40, 61) and the testis (30, 37, 62). We therefore sought initially to establish whether CREM was expressed in the ovary and whether its expression was regulated. Using several different animal models (immature female rats treated with exogenous gonadotropins or adult female rats progressing through the estrous cycle) we found that CREM is expressed in the ovary and that CREM mRNA is in-

duced by the gonadotropins, particularly hCG or LH. Our data also indicate that the inducible CREM transcripts found in the ovary correspond to mRNAs encoding the previously reported isoforms of the repressor ICER, and that ICER mRNAs are expressed in several cell types in the ovary but are selectively induced in the granulosa cells of preovulatory follicles in response to hCG or LH. We also found that ICER proteins are induced in the ovary in response to the gonadotropins and that maximal protein expression is delayed several hours beyond RNA expression, as expected. Using primary cultures of rat granulosa cells or the immortalized mouse granulosa cell line GRMO2, we demonstrate that ICER gene expression can be induced by a variety of treatments known to increase intracellular cAMP levels, and that ICER induction involves the actions of PKA, as assessed by sensitivity to the PKA inhibitor H89. Finally, there is a very strong temporal association between ICER mRNA and induction and inhibin α -subunit mRNA down-regulation in response to hCG treatment in immature rats or the preovulatory LH surge in adult cycling rats.

The above studies indicate that ICER is induced in the appropriate cell type, the granulosa cell, and at the appropriate time, after the LH surge, to play a role in mediating the down-regulation of FSH-stimulated ovarian genes such as the inhibin α -subunit gene. To test directly whether ICER is able to impact the regu-

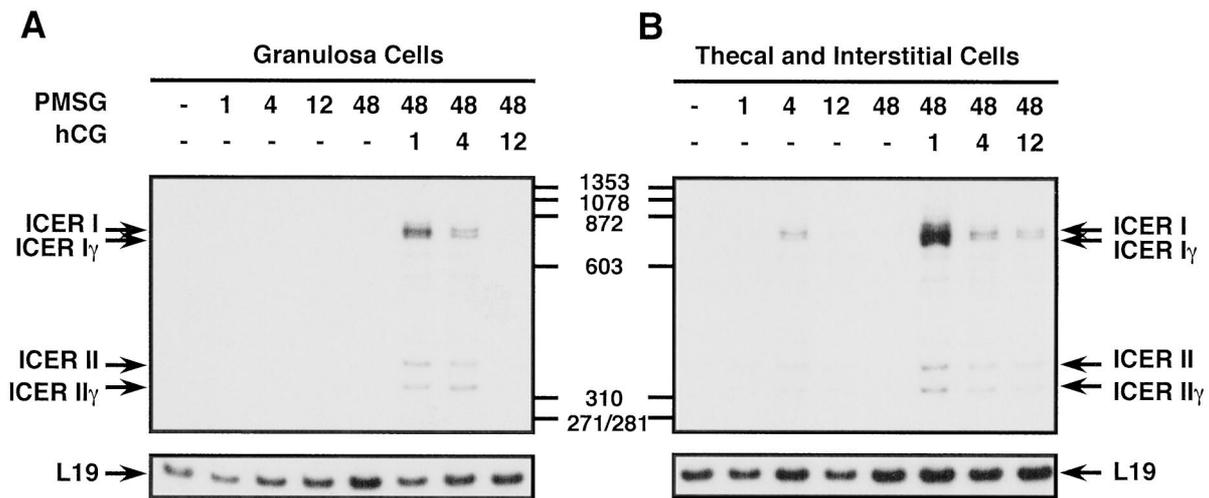


Fig. 6. Expression of ICER mRNAs in Granulosa vs. Thecal-Interstitial Cells
Immature rats were either untreated, treated with PMSG alone, or treated with PMSG and hCG for various times as indicated. Granulosa cells were isolated as described in *Materials and Methods*, and the remaining ovarian tissue was used as the thecal and interstitial cell-enriched fraction. Panel A, RT-PCR products from granulosa cell RNAs. Panel B, RT-PCR products from thecal and interstitial cell RNAs. The *arrows* indicate the positions of the four ICER isoforms I, I γ , II, and II γ . Size markers in nucleotides are shown *between the panels*. All RNA samples were also analyzed by RT-PCR for ribosomal protein L19 mRNA as an internal control (L19).

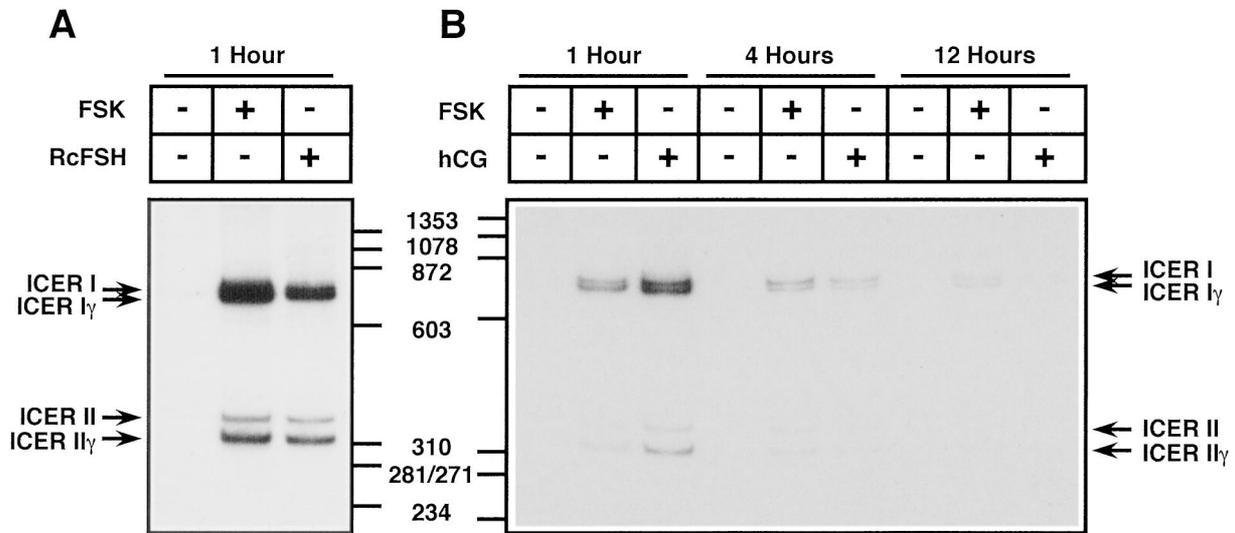


Fig. 7. Expression of ICER mRNA in Primary Cultures of Rat Granulosa Cells
Granulosa cells were isolated either from immature rats (panel A) or from immature rats treated with PMSG for 24 h (panel B). Cells were treated with 10 μ M forskolin (FSK), 1 IU/ml recombinant human FSH (RcFSH), or 0.2 IU/ml hCG for the indicated times. RNAs were analyzed by RT-PCR for ICER mRNAs and autoradiograms are shown. The *arrows* indicate the positions of the four ICER isoforms, I, I γ , II, and II γ . Size markers in nucleotides are shown *between the panels*. All RNA samples were also analyzed by RT-PCR for ribosomal protein L19 mRNA as an internal control (not shown).

lation of the inhibin α -subunit gene, we first demonstrated that ICER can interact with the nonconsensus CRE found in the inhibin α -subunit promoter using gel mobility shift and antibody supershift approaches. We next overexpressed ICER in a granulosa cell line and assessed the effect of excess ICER on inhibin α -subunit gene expression. Our finding that ICER can substantially repress both basal and cAMP-stimulated ex-

pression of this gene *in vitro* is supportive of a direct role for ICER in the regulation of inhibin α -subunit gene expression *in vivo*.

Based on our previous studies of gonadotropin-induced CREB phosphorylation in the ovary (41) and the findings presented here on gonadotropin-induced ICER expression, we can propose a model for how FSH and LH might exert their opposing actions on

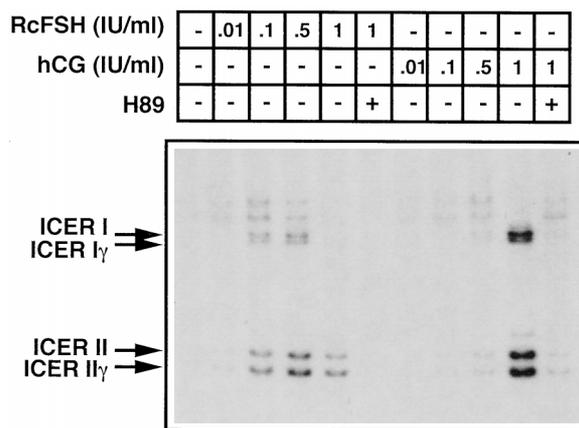


Fig. 8. Effect of the PKA Inhibitor H89 on ICER mRNA Induction by Gonadotropins in GRMO2 Cells

Mouse GRMO2 cells were treated *in vitro* with recombinant human FSH (RcFSH) or hCG at the indicated doses for 1 h. Cells treated with the highest doses of RcFSH or hCG were also simultaneously treated with 10 μ M H89, a PKA inhibitor. RNA samples from these cells were analyzed by RT-PCR for ICER mRNAs. An autoradiogram of the PCR products is shown. The four arrows indicate the positions of the four ICER isoforms, I, I γ , II, and II γ . All RNA samples were also analyzed by RT-PCR for ribosomal protein L19 mRNA as an internal control (not shown).

inhibin α -subunit gene expression in ovarian granulosa cells. During the follicular phase of the estrous cycle, FSH stimulates the proliferation of ovarian granulosa cells, and this is accompanied by increasing expression of the inhibin α -subunit gene. FSH, by stimulating intracellular cAMP production and activating PKA, also induces the phosphorylation of CREB on serine 133, making it competent to transactivate CRE-containing target genes such as the inhibin α -subunit gene (41, 42). Once ovarian granulosa cells acquire functional LH receptors, the preovulatory LH and FSH surges trigger granulosa cell differentiation and subsequent luteinization. The LH surge, through cAMP and PKA-dependent pathways, also transiently induces the transcription of the four ICER mRNAs. We propose that one function of ICER is to rapidly repress the transcription of CRE-containing and CREB-activated target genes such as the inhibin α -subunit gene. ICER itself is then down-regulated through an autoregulatory mechanism, thus completing a cycle of cAMP-dependent gene activation and repression (39).

A question of primary importance with respect to this model is why FSH and LH, both acting predominantly through cAMP-dependent mechanisms, have such divergent actions? One likely answer is provided by our data showing that FSH (PMSG) treatment induces ICER in thecal and interstitial cells, but not in granulosa cells. Whether this represents the weak LH-like activity of PMSG on luteal and interstitial cell LH receptors or a direct FSH effect is not known, but in either case ICER is not induced in granulosa cells *in vivo* by this hormonal treatment associated with gran-

ulosa cell proliferation and inhibin α -subunit mRNA induction. A second likely answer comes from studies on ICER activation by cAMP signaling. The CREM intronic P2 promoter includes four clustered CRE-like elements termed CAREs, or cAMP-autoregulatory elements (39). These elements mediate cAMP induction through activating factors such as CREB, and subsequently bind ICER, resulting in an autorepression of ICER expression and a resetting of the system. Because several of the CAREs are fairly poor consensus CRE elements, it is possible that they are occupied by activators only when the cAMP-PKA-signaling pathway is fully activated. The magnitude of the intracellular cAMP signal generated in the granulosa cells of preovulatory follicles by the LH surge is much larger than that generated by FSH in the granulosa cells of maturing follicles (19, 20), providing a potential mechanism for the selective activation of ICER by LH *in vivo*. Our studies showing that ICER expression can also be induced *in vitro* by high doses of recombinant FSH are consistent with the idea that the magnitude of the cAMP signal is a critical determinant of the ICER response.

The CREM gene has been disrupted by homologous recombination in embryonic stem cells, and the homozygous mutant mice generated from these cells lack both CREM and ICER expression (63, 64). The male mutant mice exhibit severe defects in spermatogenesis, consistent with a proposed role of CREM and ICER in this process (63, 64). Paradoxically, the female mutant mice are reported to be fertile, although detailed studies of reproductive phenotypes in the female have not yet been reported. This finding might suggest that ICER is not critical as a transcriptional regulator during LH-induced ovulation and luteinization in the rodent ovary. An alternative explanation is that other factors are able to compensate for the loss of ICER expression in the ovary. For example, similar repressor forms of the CREB gene are expressed through alternative RNA processing mechanisms in the testis (35). While we did not detect RNAs for these repressor forms of CREB in the ovary of normal animals (41), they might be induced in the ovary in the absence of ICER in the mutant animals and serve a similar function. It is also unlikely that ICER is the only factor involved in the repression of cAMP-induced gene expression in response to the LH surge. Indeed, ICER expression in granulosa cells is very transient, whereas there is an extended down-regulation of target genes such as the inhibin α -subunit gene, which remains repressed in luteinized cells in the rodent ovary (5, 65). It has recently been shown that LH treatment leads to a down-regulation of steroidogenic factor-1 (SF-1) protein in rat granulosa cells, and this appears to be an important part of the mechanism that underlies the down-regulation of rat aromatase CYP19 gene expression (66). The inhibin α -subunit gene promoter contains several consensus SF-1 binding sites, and SF-1 is likely to be involved in its regulation in granulosa cells. An additional factor that is likely to be

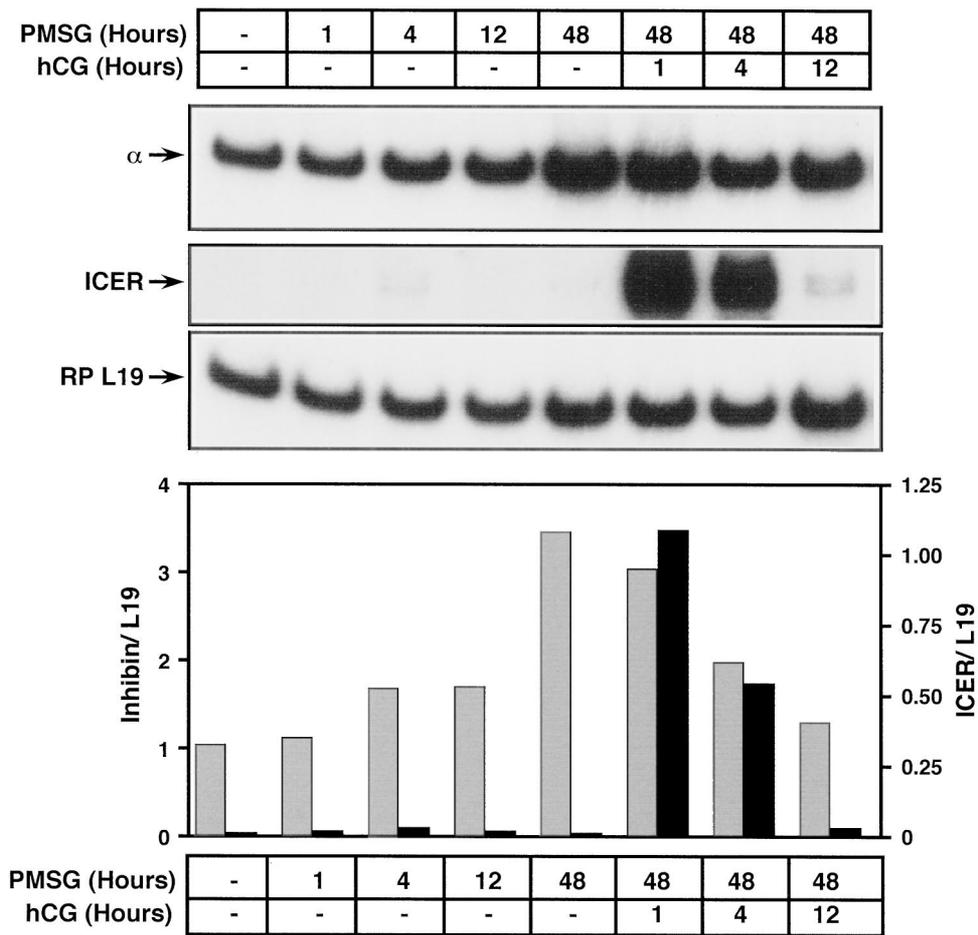


Fig. 9. Gonadotropin Regulation of Inhibin α -Subunit and ICER mRNA Expression in Ovarian Granulosa Cells

Immature rats were treated with PMSG or PMSG and hCG for the indicated times, and granulosa cells were isolated from the ovaries. Granulosa cell RNAs were analyzed by RT-PCR for inhibin α -subunit mRNA (Inhibin), ICER mRNA (ICER), and ribosomal protein L19 mRNA (RPL19) as an internal control. Autoradiograms of the RT-PCT products are shown (only the I and I γ bands of ICER are shown, but II and II γ were also analyzed). These were quantified using a PhosphorImager, and the relative levels of inhibin α -subunit and ICER mRNAs, normalized to the L19 mRNA control, are plotted in the lower panel. Similar results were obtained in several independent experiments.

important for down-regulation of cAMP-regulated genes is the bZip protein C/EBP β (67). Recent studies indicate that female mice homozygous for a disruption of the C/EBP β gene are infertile and exhibit defects in ovulation (68). Interestingly, several genes that are normally down-regulated by the LH surge, including aromatase and prostaglandin endoperoxide synthase-2 (PGS-2), fail to be down-regulated in the ovaries of the C/EBP β mutant mice (68). C/EBP β mRNA is rapidly induced by hCG in granulosa cells (66), but peak levels of C/EBP β mRNA in preovulatory follicles are not observed until 4–7 h after hormone administration (68). Thus, C/EBP β might be critical for the longer term repression of this class of genes, whereas ICER might play a more predominant role in the immediate early responses to the LH surge. Further studies of the roles of transcription factors such as SF-1 and C/EBP β in inhibin α -subunit gene expression should be informative in this regard.

The dephosphorylation of CREB attenuates its transcriptional activity (69, 70), and this represents another important mechanism for suppressing cAMP-induced transcriptional responses. However, we have previously reported that hCG rapidly stimulates CREB phosphorylation in PMSG-primed rat granulosa cells (41), suggesting that CREB dephosphorylation cannot fully explain the ability of LH to attenuate FSH-induced gene expression in granulosa cells. Furthermore, ICER mRNA induction requires CREB (71), indicating that CREB is likely to be phosphorylated and active at the time of the LH surge. Recent studies in pituitary AtT20 cells have investigated the relative contributions of CREB dephosphorylation and ICER induction in attenuating cAMP-induced transcriptional responses and also addressed the important concept of the dynamics of cAMP-induced transcriptional responses (72). These experiments and others define a refractory period after the initial stimulation of cAMP-dependent

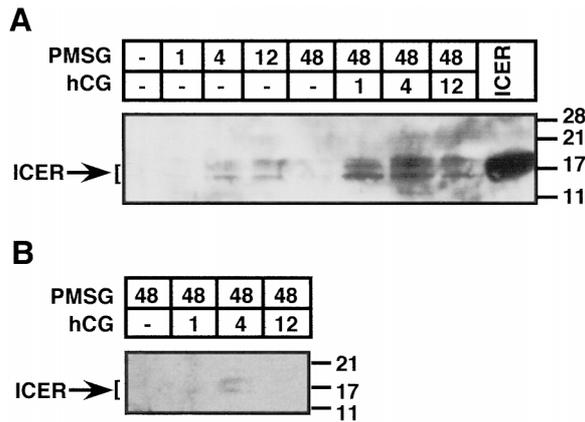


Fig. 10. Expression of ICER Protein in the Rat Ovary
 Western protein blot analysis of ICER expression in lysates from the thecal and interstitial cell-enriched fraction (panel A) or the granulosa cell-enriched fraction (panel B) of gonadotropin-stimulated rat ovaries. Immature rats were treated with hormones as indicated, and the ovaries were used to prepare granulosa and thecal-interstitial fractions as described in legend to Fig. 6. Detection was with an anti-CREM antibody that detects the ICER protein, and the ICER proteins are indicated by the *bracket*. The positions of mol wt standards are also indicated. In Panel A, 5 μ g of lysate from HeLa T4 cells transfected with an ICER I expression clone, as described in *Materials and Methods*, were used as positive control (ICER).

genes that is determined in part by the duration of the initial stimulus (73, 74). These findings may have strong parallels in the ovary that would help to explain our data showing that while the primary gonadotropin surges on proestrus evening strongly induce ICER expression, the secondary FSH surge early on the morning of estrus does not. The intense and sustained primary LH surge that induces ICER expression might, in addition to initiating the events that lead to target gene repression, serve to make granulosa cells refractory to further ICER induction in response to the secondary FSH surge, thus allowing FSH to act in a stimulatory fashion to induce the gene expression and cell proliferation events that are critical to the recruitment and maturation of a new cohort of ovarian follicles. It seems reasonable to speculate that cycles of gonadotropin-induced and cAMP-mediated induction and attenuation of transcriptional responses may be a key mechanistic component for maintaining the cycles of ovarian follicular development characteristic of reproduction in many mammals.

MATERIALS AND METHODS

Animals and Hormone Treatments

Immature 21- to 23-day-old female Sprague-Dawley rats (Harlan Breeding Laboratories, Indianapolis, IN), were kept on 14-h, 10-h light-dark cycles with lights on at 0500 h. Rats were injected with PMSG (10 IU, sc, Sigma, St. Louis, MO) for

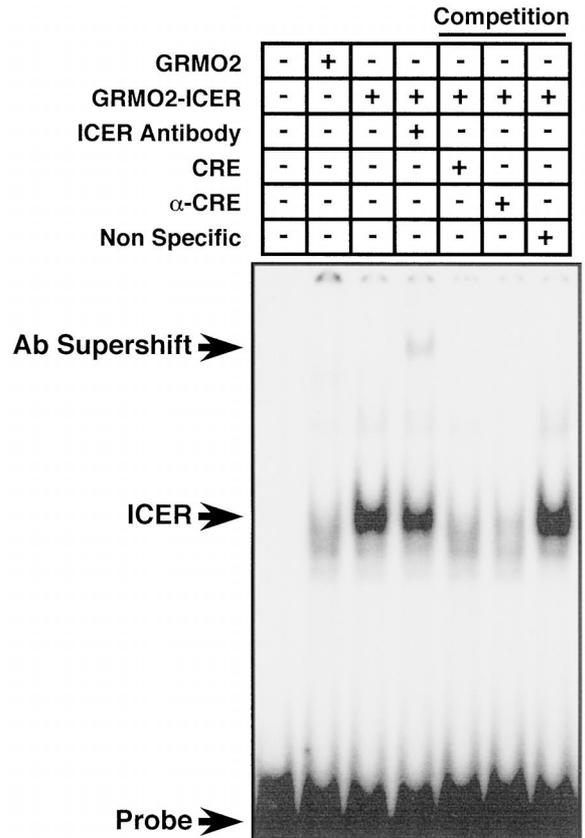


Fig. 11. Interaction of ICER with the Inhibin α -Subunit CRE
 Whole-cell extracts (5 μ g) from GRMO2 cells (GRMO2) or vaccinia T7-infected GRMO2 cells transfected with ICER I expression clone (GRMO2-ICER) were incubated with a 32 P-labeled double-stranded oligonucleotide probe that spans the inhibin α -subunit CRE. The binding reaction was incubated with either an anti-CREM/ICER antibody (ICER Antibody) or with a 100-fold excess of a consensus CRE (CRE), the α -inhibin CRE (α -CRE), or a nonspecific competitor DNA. 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 ICER*. The antibody supershifted complex is indicated by an *arrow labeled Ab supershift*, and the free probe (*bottom of gel*) is indicated by an *arrow labeled probe*.

up to 48 h and later with hCG (10 IU, ip, Sigma) for up to 12 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 for later use. Adult female Sprague Dawley rats (body weight 150–180 g, Charles Rivers Breeding Laboratories, Wilmington, MA) were maintained as described above. Estrous cycle stages were determined by daily examination of vaginal cytology. Only animals that showed at least two consecutive 4-day cycles were used. Rats were killed and ovaries removed at 0900 and 1800 h on each day of the cycle as well as at 1200, 1400, 1600, 2000, and 2200 h on proestrus and 0200 h on estrus. Ovaries were frozen rapidly on dry ice and stored at -80 C until use. Trunk blood was collected for gonadotropin measurements. FSH and LH were determined by RIA using rat FSH-RP-2 and LH-RP-3 from NIDDK as standards (75). Intra- and interassay coefficients of variation were less than 10% in both assays. For antagonist experiments, rats were

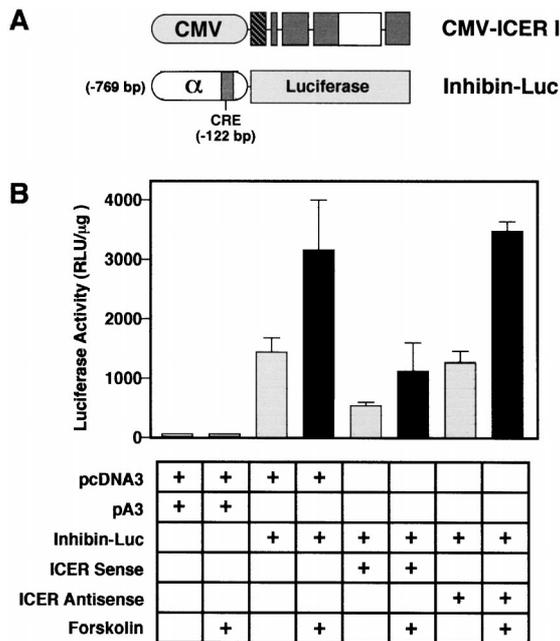


Fig. 12. Effects of ICER Overexpression on Inhibin α -Subunit Promoter Activity in GRMO2 Cells

Panel A, The structures of the CMV-ICER I expression construct and the inhibin α -subunit-luciferase reporter gene used for this experiment are shown. The exons of ICER I correspond to those illustrated in Fig. 1. The inhibin α -subunit reporter (inhibin-Luc) contains a CRE element in the promoter at -122 bp. Panel B, Luciferase activity in GRMO2 cells transiently cotransfected with the indicated DNAs. pA3 is the parental plasmid of inhibin-Luc and was used to normalize DNA amounts. ICER sense and antisense indicate the correct and reversed orientations of CMV-ICER I. Cells were transfected for 6 h, allowed to recover for 12 h, treated with 10 μ M forskolin or vehicle for 4 h, and then harvested for luciferase assay. Values are relative luciferase activity per μ g of cellular protein.

injected with pentobarbital (40 mg/kg in saline, ip, Sigma) or the GnRH antagonist WY-45760 (100 μ g/rat in sesame oil, sc, Wyeth Laboratories, Philadelphia, PA) at 1330 and 1200 h, respectively, on proestrus. Rats treated with vehicle alone served as controls, and animals were killed for analysis at 1800 h proestrus.

Granulosa Cell Cultures

Immature 21- to 23-day old female rats with or without PMSG treatment (10 IU, sc, for 24 h) were used for granulosa cell preparation utilizing follicular puncture essentially as described (42, 76). Ovaries were collected into serum-free medium (4F), which consists of 15 mM HEPES, pH 7.4, 50% DMEM, and 50% Ham's F12 with transferrin (5 μ g/ml), human insulin (2 μ g/ml), hydrocortisone (40 ng/ml), and antibiotics. After incubating the ovaries at 37 C in 4F medium containing 0.5 M sucrose and 10 mM EGTA for 30 min, the ovaries were washed in fresh 4F medium. Individual follicles were punctured, and the granulosa cells were extruded using a 23 ga needle under a Reichert dissection microscope (Buffalo, NY). Cells were plated in 4F medium supplemented with 10% FBS (GIBCO BRL, Grand Island, NY), and incubated in a humidified atmosphere of 5% CO₂ at 37 C. Cultured cells were either untreated or treated *in vitro* with 10

μ M forskolin (Sigma), varying doses of hCG, or recombinant human FSH (line 1, sample 329, NICHD).

Isolation of CREM and ICER cDNAs

Rat CREM cDNAs were cloned from total testicular RNA by RT-PCR using oligonucleotide primers 1 and 2 (see Fig. 1) corresponding to nucleotide positions 15-36 and 1115-1135, respectively, of the mouse CREM cDNA (27). DNA sequence analysis indicated that cDNAs encoding the τ and τ 2 isoforms of CREM protein were obtained. Total RNA isolated from the ovary of an adult rat at 1800 h proestrus was used to clone ICER cDNAs by RT-PCR. Oligonucleotide primer 4 corresponding to nucleotides 1-21 of ICER cDNA (39) and oligonucleotide primer 2 were used for PCR amplification. All oligonucleotide primers were purchased from Cruachem (Sterling, VA). The ICER-I expression construct was made by cloning the ICER-I RT-PCR product (see Fig. 1) into the expression vector pcDNA-3 (Invitrogen, San Diego, CA), which includes the human cytomegalovirus (CMV) promoter and 3'-polyadenylation sequences from the bovine GH gene.

RNA Isolation and Analysis

RNA from ovaries or granulosa cells was isolated by homogenization in 4 M guanidine isothiocyanate containing 25 mM sodium citrate, 0.5% sarkosyl, and 7 μ M β -mercaptoethanol and extraction with acid-phenol (77) or by centrifugation through CsCl gradients. For RT-PCR assays, total ovarian RNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase in the presence of deoxynucleosidetriphosphates (1 mM) and random hexameric oligonucleotides, and aliquots of this cDNA were amplified by PCR with incorporation of [³²P]dCTP into the PCR product as described previously (78). PCR primers 2 and 4 (see Fig. 1) and primers specific for the inhibin α -subunit (79) were used. Rat ribosomal protein L-19 (80) was used as an internal control in all experiments. The PCR products were separated by size using electrophoresis on 5% polyacrylamide gels, the gels were dried, and PCR products were visualized by autoradiography on X-OMAT-AR film (Eastman Kodak Company, Rochester, NY) and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For Northern RNA blot analysis, 20 μ g total RNA were separated by size using electrophoresis in a 1.2% agarose gel containing 1 \times 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer and 6% formaldehyde (81) and visualized by acridine orange staining. Size-fractionated RNA was transferred to Biotrans Nylon membrane (ICN, Irvine, CA), immobilized on the membrane by UV cross-linking and baking at 80 C for 30 min. A 47-nucleotide antisense oligonucleotide, primer 3 (see Fig. 1) corresponding to nucleotide positions -1 to -47 (39) unique for ICER was end-labeled with [³²P]dCTP and terminal deoxynucleotide transferase and used as the hybridization probe. The blots were washed at a final stringency of 0.1 \times saline sodium citrate (SSC)/0.01% SDS at 65 C. ICER mRNA was visualized by autoradiography on X-OMAT-AR film (Eastman Kodak). Radioactive signal from the blot was removed by washing in 10 mM sodium phosphate (pH 6.5) in 50% formamide at 65 C for 1 h, followed by 2 \times SSC/0.1% SDS at room temperature for 15 min. The blot was then hybridized to a ribosomal protein S2 (82) probe, prepared by random priming, as a control to compare the amounts of RNA loaded and transferred.

In Situ Hybridization

Sections (20- μ m) of frozen ovaries were prepared using a Reichert 820 cryostat and mounted onto gelatin and poly L-lysine-coated glass slides for *in situ* hybridization as described previously (78). Hybridization probes used were

[³⁵S]UTP or [³³P]UTP-labeled riboprobes derived from the full-length $\tau 2$ CREM cDNA clone, a 5'-NcoI subclone (see Fig. 1) of rat CREM $\tau 2$, a rat LH receptor cDNA subclone, or a [³⁵S]dATP-labeled 47-nucleotide long antisense oligonucleotide that is unique to ICER isoform (primer 3 in Fig. 1). Hybridization was continued for 12–18 h at 47 C in a humidified chamber. Sense riboprobes were used as controls. Subsequently, the slides were washed to a final stringency of $0.1 \times$ SSC at 65 C after a 1-h treatment with 20 μ g/ml RNase at 37 C. Slides were then processed for emulsion autoradiography (NTB-2, Eastman Kodak). Exposure time on emulsion was 2 weeks. After development of the slides, they were stained with hematoxylin to visualize nuclei. The sections were then examined and photographed using a macroscope (Wild M420, Leica, Heerbrugg, Switzerland) or a Nikon Optiphot microscope (Nippon Kogaku, Inc., Garden City, NY).

Preparation of Protein Extracts and Western Blot Analysis

Protein lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, leupeptin, aprotinin, 1 mM NaF). For cultured cells, after the cells were washed with PBS and collected by low-speed centrifugation, they were resuspended in lysis buffer and incubated on ice for 10 min and then lysed by two cycles of freezing and thawing. The lysates were centrifuged to remove nuclear debris, and the supernatant was collected and frozen at -80 C until further use. Frozen tissue samples were pulverized in dry ice and homogenized in lysis buffer using six to eight strokes with a Dounce homogenizer. This homogenate was subjected to two cycles of freezing and thawing and processed as described above. Protein concentrations were estimated using a Bradford colorimetric assay (Bio-Rad, Richmond, CA).

Protein lysates (100 μ g) were boiled for 5 min in denaturing sample buffer and size separated on a 10–18% continuous gradient SDS-polyacrylamide gel, and proteins were transferred to nitrocellulose (BA-85, Schleicher & Schuell, Keene NH). The membrane was washed with water and blocked with 3% nonfat dry milk in PBS (blocking buffer) for 30 min at room temperature with shaking. The blot was then incubated with primary antibody (anti-CREM at 1:250) in blocking buffer for 48 h at 4 C with gentle shaking. The blot was washed two times with water and once with PBS followed by a 45-min incubation at room temperature with goat antirabbit antibody conjugated to horseradish peroxidase (Promega, Madison WI) in blocking buffer. The blot was then washed twice with water, once with PBS, and once with PBS containing 0.05% Tween 20. The blot was rinsed with water, and antibody-antigen complexes were visualized using an enhanced chemiluminescence system (ECL kit, Amersham, Little Chalfont, Buckinghamshire, U.K.).

GRMO2 Cell Culture, Transfection, and Luciferase Assays

GRMO2 cells (provided by N.V. Innogenetics, Ghent, Belgium) were cultured as described (47, 48) in HDTIS (DMEM-F12 1:1, 10 μ g/ml insulin, 5 nM sodium selenite, 5 μ g/ml transferrin) supplemented with 2% FBS and sodium pyruvate (100 mg/liter) in a humidified incubator at 37 C and 5% CO₂. GRMO2 cells were either untreated or treated *in vitro* with 10 μ M forskolin, varying doses of hCG, or recombinant human FSH with or without the PKA inhibitor H89 (Sigma). Cationic liposomes, prepared as described previously (83), were used for transient transfection of GRMO2 cells with DNA (84). Plasmid DNA (2.5 μ g) for each well of a 12-well culture dish was incubated at room temperature with lipofection reagent for 20–30 min in OptiMEM and added to cells washed with PBS. After 6 h, the DNA-lipid mixture was

replaced with fresh HDTIS containing 2% FBS, and the cells were incubated for 12 h. Fresh medium, or medium containing 10 μ M forskolin, was then added to the cells. After 4 h of hormone treatment, the cells were washed with PBS and lysed by gently agitating on ice in sample buffer (25 mM HEPES, pH 7.8, 15 mM MgSO₄, 1 mM dithiothreitol, 0.1% Triton X-100). Luciferase assays were performed essentially as described (85). Cell lysates (100 μ l) were added to 400 μ l of assay buffer (25 mM HEPES, pH 7.8, 15 mM MgSO₄, 5 mM ATP, 1 μ g/ml BSA), and 100 μ l of 1 mM luciferin (sodium salt) (Analytical Bioluminescence, San Diego, CA) were added using an automatic injector; emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence) for 10 sec. Cell lysates (20 μ l) were used for total protein determination using the Bio-Rad protein assay reagent. For Western blot analysis and electromobility shift assays, the vaccinia-T7 RNA polymerase hybrid expression system (50) was used. GRMO2 or 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 30–40 min in PBS/0.1% BSA. Virus was aspirated, and pre-incubated DNA-liposome mixture was added to the cells and incubated for 12 h. Cells were immediately harvested and processed for protein isolation.

Electrophoretic Mobility Shift Assays

A 20-bp double-stranded oligonucleotide probe that spans the inhibin α -subunit CRE sequence was end labeled using [³²P- γ]ATP and T4 polynucleotide kinase (Promega, Madison, WI) and purified using a G-25 spin column (Boehringer Mannheim GmbH, Mannheim, Germany). Whole-cell lysates (5 μ g) from vaccinia-infected transfected cells were used for each binding reaction. Whole-cell lysates, prepared as described earlier in this section, were incubated in binding buffer [50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 10% glycerol (vol/vol), 0.5 mM dithiothreitol, 50 ng/ μ l poly deoxy(inosinic-cytidylic)acid] for 20 min at room temperature with 1 ng of ³²P-labeled oligonucleotide probe. Where indicated, 100 ng of double-stranded unlabeled competitor oligonucleotide were added to the binding reaction 5 min before the addition of probe. For antibody supershift studies, 1 μ l of anti-CREM antibody was added to the reaction, and the reaction was incubated at room temperature for an additional 20 min. 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).

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