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# Structure of the Rat Inhibin and Activin $\beta_A$-Subunit Gene and Regulation in an Ovarian Granulosa Cell Line*

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**ABSTRACT**

We have isolated the rat inhibin and activin $\beta_A$-subunit gene, which is composed of three exons, and have characterized a 571-bp region upstream from the transcriptional start site that functions as a promoter in transient transfection studies in an ovarian granulosa cell line, GRMO2. Deletion analysis of the 571-bp promoter region has identified DNA sequences between −362 bp and −110 bp to be essential in mediating basal promoter activity and activation by forskolin (FSK) and/or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Within this region, a variant CRE (cAMP response element) has been identified at −120 bp. Point mutations in the variant CRE substantially reduce the ability of FSK and/or TPA to induce promoter activity in GRMO2 cells. A single nucleotide change in the variant CRE, which converts it to a consensus CRE, does not enhance promoter activity in response to FSK and/or TPA, but rather reduces promoter activity to the same extent as the other inactivating mutation in the variant CRE, suggesting that this element does not act as a classical CRE. Consistent with this, electrophoretic mobility shift assays performed using antibodies to a variety of cAMP and phorbol ester-responsive transcription factors indicate that the AP-1 family proteins jun-B and fos-B are present in the protein complex binding to the variant CRE. Overexpression of jun-B and fos-B in GRMO2 cells resulted in a robust activation of the $\beta_A$-subunit promoter. Our results suggest that this novel variant CRE sequence mediates both cAMP and phorbol ester regulation through its interactions with AP-1 family proteins. (*Endocrinology* **139:** 3271–3279, 1998)

INHIBIN and activin are protein hormones that belong to the transforming growth factor-$\beta$ (TGF-$\beta$) superfamily of proteins, whose members are known to participate in diverse biological functions affecting cell proliferation and differentiation (1). One of the major physiological roles attributed to inhibin and activin is regulation of the reproductive axis, which derives from their ability to suppress or activate, respectively, the synthesis and secretion of pituitary FSH (2–4). Inhibin and activin are produced by the combinatorial assembly of three related protein subunits ($\alpha$, $\beta_A$, and $\beta_B$). Inhibin A and inhibin B are formed by heterodimerization of the $\alpha$-subunit and one of the two $\beta$-subunits, whereas the three forms of activin are formed by dimerization of the highly related $\beta$-subunits. The molecular cloning of the $\alpha$, $\beta_A$, and $\beta_B$ complementary DNAs (cDNAs) has revealed that each subunit is encoded by a separate gene and is derived from the carboxyl-terminus of a larger precursor protein (5–8).

In the rodent ovary, messenger RNAs (mRNAs) for the $\alpha$-, $\beta_A$-, and $\beta_B$-subunit genes are expressed predominantly in the granulosa cells of healthy follicles, and their abundance increases during follicular maturation and decreases in preovulatory follicles following the proestrous LH surge (9, 10). In the adult rat, FSH induces the expression of the $\alpha$-, $\beta_A$-, and $\beta_B$-subunit genes in preovulatory follicles (11, 12). FSH also stimulates inhibin secretion and $\alpha$-and $\beta$-subunit mRNA accumulation in cultured primary granulosa cells (9, 13). Forskolin (FSK), a pharmacological agent that mimics the activity of FSH by activating adenylyl cyclase and increasing intracellular levels of cAMP, also stimulates inhibin gene expression and secretion in granulosa cells, suggesting a role for cAMP in the regulation of $\alpha$- and $\beta$-subunit gene expression (11, 14). To explore the role of cAMP in the regulation of inhibin and activin gene expression, we and others reported earlier the isolation and structural characterization of the rat inhibin $\alpha$-subunit gene. We identified a cAMP response element (CRE) in the promoter of the $\alpha$-subunit gene that is necessary for mediating gonadotropin and cAMP regulation of the gene in rat granulosa cells (15–17). We, and others, also characterized the rat inhibin and activin $\beta_B$-subunit gene and found that, while it is structurally similar to the $\alpha$-subunit gene, it is not regulated by cAMP in transfected gonadal cells (14, 18). We have now extended those studies and report the isolation of the rat inhibin and activin $\beta_A$-subunit gene and characterization of its promoter region. To assess the regulation of this gene, we use a recently characterized immortalized granulosa cell line, GRMO2, that retains many of the characteristics of ovarian granulosa cells. Using transient transfection assays, we identify a variant CRE element as being critical for regulation of this gene by both cAMP and phorbol esters, and demonstrate that AP-1 family proteins play an important role in the regulation of $\beta_A$-subunit gene transcription.

## Materials and Methods

### *Cloning of the inhibin and activin $\beta_A$ gene and construction of luciferase fusion genes*

Rat genomic libraries (one constructed with liver DNA in λ Charon 4A, the other with HTC hepatoma cell DNA in λ-Fix, Stratagene (La Jolla, CA) (19) were screened with probes from the rat $\beta_A$-subunit cDNA clone rINB-5 (8). The 5′ probe was a 240-bp *Eco*RI/*Bam*HI fragment that

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contained untranslated sequences and the first 24 amino acids of the $\beta_A$ prohormone. The 3′ probe was a 510-bp *Pst*I/*Eco*RI fragment spanning the mature $\beta_A$-subunit sequences. No plaques that hybridized to both probes were found; however, a 5′-positive phage was purified from the λ-Fix library, and a 3′-positive phage was purified from the Charon 4A library. Southern blotting of these two clones identified a 4.3 *Bam*HI fragment and a 6.1-kb *Eco*RI fragment that hybridized to the 5′ and 3′ probes, respectively. These two fragments, as well as the 0.5 kb *Bam*HI fragment immediately 3′ of the 4.3-kb *Bam*HI fragment, were subcloned into pGEM vectors. Subsequent Southern DNA blotting, subcloning, dideoxynucleotide chain termination DNA sequencing (Sequenase, U.S. Biochemical Corp., Cleveland, OH) and primer extension and S1 nuclease analysis (described below) revealed that these three genomic DNA clones, in combination, contain three exons comprising the $\beta_A$-subunit mRNA as well as approximately 1.5 kb of 5′ flanking sequences. To construct the initial fusion gene, a 609-bp *Nae*I fragment extending from −571 to +38 bp was ligated into a *Sma*I site adjacent to the luciferase coding region of the vector pA3-Luc (kindly provided by Dr. William Wood, University of Colorado Health Sciences Center, Denver, CO) (20). 5′ deletions extending to an *Acc*I site at −362 bp and a *Hin*fI site at −110 were made, and these three constructs were designated (−571)r$\beta_A$-Luc, (−362)r$\beta_A$-Luc, and (−110)r$\beta_A$-Luc.

### *Analysis of the $\beta_A$-subunit mRNA structure*

The $\beta_A$ gene transcriptional start site was determined by combining S1 nuclease mapping and primer extension analysis. For S1 nuclease mapping, probes S1–1 and C were 3′-end-labeled with [$\gamma^{32}$P]ATP (DuPont NEN, Boston, MA) and $T_4$ polynucleotide kinase. Approximately $1 \times 10^5$ cpm/ng probe was hybridized to 5 μg of polyA$^+$ ovarian RNA (or liver RNA as a control) in 20 μl of formamide hybridization buffer (80% formamide, 40 mM PIPES (piperazine-*N*,*N*′-bis[2-ethane-sulfonic acid), pH 6.8, 400 mM NaCl, and 1 mM EDTA. Hybridization was initially at 80 C for 5 min, then at 35–55 C for 12–16 h. Three hundred microliters of 1500 U/ml S1 nuclease (Gibco BRL, Gaithersburg, MD) in S1 salts buffer (0.28 M NaCl, 50 mM NaOAc (pH 4.5), 4.5 mM ZnSO4, 20 μg/ml yeast transfer RNA) were added, and S1 nuclease digestion was allowed to proceed for 1.5 h at 15–22 C. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, and resuspended in formamide loading buffer. Protected fragments were resolved on 6% polyacrylamide/50% urea denaturing gels and compared with adjacent sequencing ladders to establish their size. Primer extension analysis was performed using a synthetic 30-bp oligonucleotide (5′-CCAAAGCAAGGGCATCCTGGCAGCAAAAGT-3′) complimentary to exon 2 sequences. The oligonucleotide was kinased as described above and $1 \times 10^5$ cpm/ng primer was hybridized to 5–20 μg of polyA$^+$ ovarian RNA (or liver, placenta, testis RNA as a control) in 20 μl of 100 mM KCl. Hybridization was performed at 80 C for 5 min followed by 12–16 h at 55 C. The extended product was synthesized by adding 20 μl of 2× extension mix (100 mM Tris base (pH 8.0), 20 mM MgCl, 20 mM dithiothreitol (DTT), 10 mM each of dGTP, dATP, dTTP, and dCTP and 1 U RNasin/ml (Promega, Madison, WI) and 30 U of AMV reverse transcriptase (Promega) and then incubated at 42 C for 1.5 h. One milliliter of 0.5 M EDTA was added to stop the reaction. Samples were extracted with phenol/chloroform/isoamyl alcohol, ethanol precipitated, resuspended in 2 μl of 0.1 M NaOH, and combined with 4 μl of formamide loading buffer. Gel electrophoresis was performed as described above, and parallel sequencing reactions were used to size the extended products.

### *GRMO2 cell culture, transfections, and luciferase assays*

GRMO2 cells (kindly provided by Innogenetics N.V., Ghent, Belgium), were maintained in Ham's F-12/DMEM with 2% FCS and supplemented with 10 μg/ml transferrin, 5 μg/ml insulin, 30 nM sodium selenite, 3 μg/ml BSA, and antibiotics as described previously (21). Transfection of GRMO2 cells was performed at 50–70% cell confluency using a modified lipofectin-mediated method and 1.25 μg of the relevant DNAs (22). After 6 h at 37 C, cells were washed and incubated overnight. Cells were treated with FSK (10 μM) and/or TPA (30 nM) for 24 h before harvest and assayed for luciferase reporter gene activity as described previously (23).

### *Site-directed mutagenesis*

To construct plasmids containing promoter point mutations, oligonucleotide-directed mutagenesis was performed (24). An *Nae*I/*Kpn*I fragment (−571 bp to −62 bp) from the $\beta_A$-subunit promoter was subcloned into pGEM3Zf(-) (Promega) and transformed into the *dut*$^-$ *ung*$^-$ *Escherichia coli* strain RZ1032. Uracil-enriched single-stranded phagemid was produced by growing a single colony in Terrific Broth supplemented with 5 mg/ml uridine and 100 μg/ml ampicillin for 3 h, infecting with 100 ml R408 helper phage ($1 \times 10^{11}$ pfu/ml), and continuing growth for 6 h. Collected phagemid particles were resuspended in TE, and the phagemid DNA was obtained after phenol/chloroform extraction. The oligonucleotide primer used for constructing (mCRE)r$\beta_A$-Luc had the sequence (5′-AATCAGCATGATCT-CAGCAGATGA-3′). The oligonucleotide primer used to generate the (cCRE)r$\beta_A$-Luc, had the sequence (5′-AATCAGCATGACGTCAGCAGATGA-3′). The primers were phosphorylated with T4 polynucleotide kinase and annealed to phagemid DNA in annealing buffer (40 mM Tris base, pH 7.5, 0.1 M NaCl, 20 mM $MgCl_2$ and 5 mM DTT). Primer extension and ligation were performed using Klenow and $T_4$ DNA ligase in extension buffer (20 mM Tris base, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM each of dGTP, dATP, dTTP, and dCTP, and 0.5 mM ATP). The reaction proceeded on ice for 30 min, at room temperature for 4 h, and then was used to transform *Escherichia coli* strain JM109. Several colonies were screened by DNA sequencing, and the correct mutation was subcloned as an *Acc*I/*Kpn*I fragment into $\beta_A$Nae-Luc to generate the (cCRE)r$\beta_A$-Luc and (mCRE) r$\beta_A$-Luc mutant plasmid constructs.

### *Electrophoretic mobility shift assays*

An oligonucleotide probe corresponding to the −106 bp to −135-bp region in the $\beta_A$-subunit promoter (5′-GAGTCATCTGCTGATGTCAT-GCTGATTCTA-3′) was end-labeled using $T_4$ kinase and [$\gamma^{32}$P]ATP. The probe was heated to 95 C for 5 min in the presence of a complementary oligonucleotide and annealed gradually by cooling to room temperature in 0.1 M NaCl solution. Oligonucleotides used in competition assays were similarly annealed to their complementary strands to form double-stranded probes. Binding reactions were carried out in the presence (100×) or absence of competitors (wild-type, mutant oligonucleotides, or oligonucleotides containing consensus TRE or CRE sites). The labeled probes ($10^4$ cpm, approximately $10^5$ cpm/ng) were incubated with 10 μg of nuclear extract and 1 μg of poly (dI-dC) in a buffer containing (final concentration) 10 mM HEPES (pH 7.8), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA, and 10% glycerol. The reaction mixture, in a volume of 20 μl, was incubated on ice for 20 min, and DNA-protein complexes were separated from unbound probe on a 5% polyacrylamide gel electrophoresed in 0.5× TBE (90 mM Tris borate, pH 8.2, 2.5 mM EDTA at 4 C for 2 h at 200 V. Antibody incubations with 1 μg of anti-CREB (kindly provided by Dr. Joel F. Habener, Harvard Medical School, Boston, MA), anti-ATF-1, anti-ATF-2, anti-jun-B, anti-jun-D, anti-c-jun, anti-c-fos, anti-fos-B or anti-fra-2 (Santa Cruz Biotechnology, Santa Cruz, CA), were performed before binding for 45 min at room temperature.

### *Statistical analysis*

Data for transfection and cotransfection studies were expressed as the mean ± SEM; n = 3. Statistical analyses were performed using unpaired student's *t* test. A two-tailed probability of less than 5% (*i.e.* $P < 0.05$) was considered statistically significant.

## Results

### *Cloning and structural characterization of the rat inhibin and activin $\beta_A$-subunit gene*

Genomic clones were isolated by screening a rat genomic library with probes from both the 5′ and 3′ ends of the rat $\beta_A$-subunit cDNA clone rINB-5 (8). A combination of restriction enzyme mapping, Southern DNA blotting, subcloning and sequencing revealed that the clones rINBgen-5′ and rINBgen-3′, and a 0.5 kb *Bam*HI fragment, in combination, con-

tained all of the protein coding sequences of the $\beta_A$ gene. Figure 1 depicts the structure of the rat $\beta_A$ gene.

The $\beta_A$ transcriptional start site was determined by primer extension analysis and S1 nuclease mapping. The probes (B and S1–1) used in these assays are represented schematically in Fig. 2A. Comparison of the sizes of the extended product (193 nt, Fig. 2B) and S1-protected fragment (189 nt, data not shown) revealed the existence of an additional intron (between nucleotides 52 and 53 of rINB-5) and untranslated exon in the $\beta_A$ gene. To establish if this exon was present in clone rINBgen-5′, an *Eco*RI/NIaIV fragment consisting of the first 40 bp of rINB-5 was used to probe an rINBgen-5′ DNA blot (data not shown). A presumptive first exon was found to be in rINBgen-5′ approximately 2.5 kb upstream of the first protein-coding exon (Fig. 1). A second S1 nuclease experiment (Fig. 2C), performed with a probe specific for the untranslated exon (C in Fig. 2A), mapped the transcriptional start site to the same A nucleotide as the previous primer extension analysis (Fig. 2A and Fig. 3A). These results indicate that the newly identified 5′ exon is 66 bp in length. The identification of this small additional exon containing only 5′ noncoding sequences of the inhibin and activin $\beta_A$-subunit gene was reported previously in preliminary form by our laboratory (25), and recent studies on the human $\beta_A$ gene indicate that it has an analogous noncoding exon (26).

To characterize the putative $\beta_A$-subunit promoter and identify *cis*-acting elements that might mediate the transcriptional regulation of the $\beta_A$ gene, 761 bp of the 5′ flanking region was subcloned and sequenced. Figure 3A shows the DNA sequences of the 5′ flanking region of the rat $\beta_A$ gene and identifies a number of consensus and near-consensus binding sites for selected transcription factors. A TATA box and a CCAAT box are located at −30 bp and −62 bp with respect to the transcriptional start site. Within the 571 bp promoter region of the rat $\beta_A$ gene, a variant CRE (cAMP-response element) site at −120 bp, two variant TREs (TPA-responsive elements) at −150 bp and −197 bp and a consensus TRE site at −537 bp are identified with respect to the transcriptional start site. Figure 3B shows the schematic representation of the $\beta_A$ promoter region and the plasmid constructs containing point mutations at the variant CRE site (to be discussed in the following section).

*Activity of the rat inhibin and activin $\beta_A$ promoter in GRMO2 cells*

To analyze the promoter activity of the $\beta_A$-subunit gene 5′-flanking sequences and delineate elements mediating regulation of the gene, luciferase fusion constructs containing 5′-flanking sequences were generated and used to transiently transfect GRMO2 immortalized mouse granulosa cells that expresses the endogenous $\beta_A$-subunit gene (21). Because potential TRE and CRE sites were found in the 5′-flanking region and the endogenous $\beta_A$-subunit gene has been shown to be regulated by phorbol esters and cAMP (27, 28), we tested responses to the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) and to the adenylate cyclase activator FSK. Figure 4 shows a significant activation of the reporter gene in response to TPA or FSK treatment when $\beta_A$ promoter regions to −571 bp and −362 bp were used. Although the (−362)r$\beta_A$-Luc reporter construct does not include the consensus TRE at −537 bp, it remained fully responsive to TPA. We also examined the effects of FSK and TPA in combination on $\beta_A$-subunit promoter in GRMO2 cells. As shown in Fig. 4, the two agents in combination give a response that is larger than that observed with either FSK or TPA alone but is less than additive. When a $\beta_A$ promoter region deleted to −110 bp was tested, a dramatic and significant decrease in both basal and inducible expression was observed. Because deletion construct (−110)r$\beta_A$-Luc removes a variant CRE at position −120 bp and a nonconsensus CRE at a similar position in the $\alpha$-subunit gene is critical for cAMP regulation, we generated two constructs bearing mutations in the CRE region in the context of the −571 bp promoter. These include a mutation that was expected to disrupt CRE function (TGATGTCA→TGA**GA**TCA) and a mutation that converted the variant CRE into a consensus, palindromic CRE (TGATGTCA→TGA**C**GTCA). Figure 5 demonstrates that in GRMO2 cells, the basal activity and FSK or TPA-inducibility of the (mCRE)r$\beta_A$-Luc construct was significantly reduced compared with the wild-type −571-bp

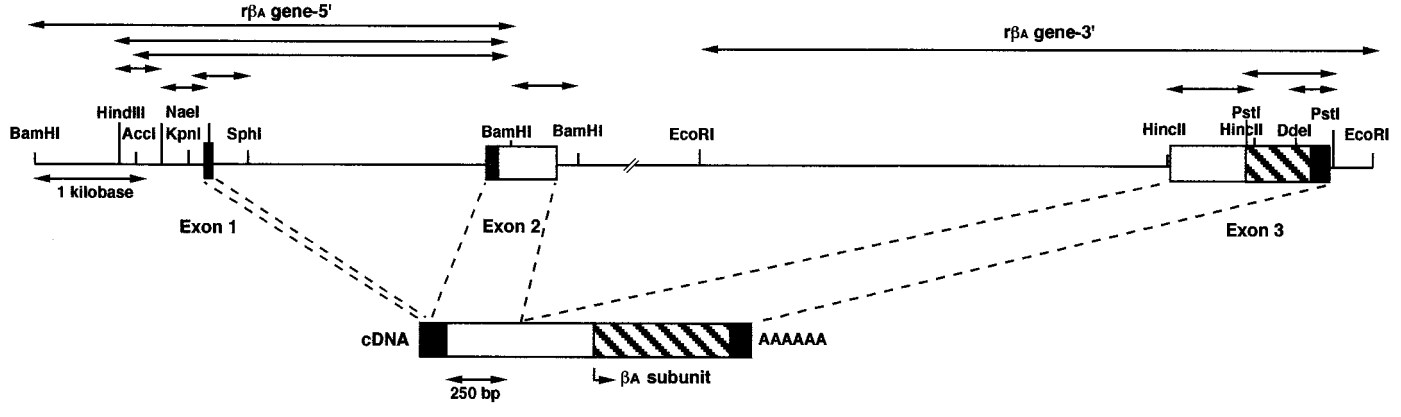


FIG. 1. Schematic structure of the rat inhibin and activin $\beta_A$-subunit gene. *On the top line*, the exon sequences are represented by *boxes*: *open boxes*, precursor protein sequences; *striped boxes*, mature $\beta_A$ chain sequences; *black boxes*, 5′ -and-3′-nontranslated regions. The restriction enzyme sites that were used for subcloning are indicated at the top. The *double-headed arrows* represent restriction fragments that were subcloned for DNA sequence analysis. On the *lower line*, the structure of the rat $\beta$-subunit cDNA is shown for comparison.

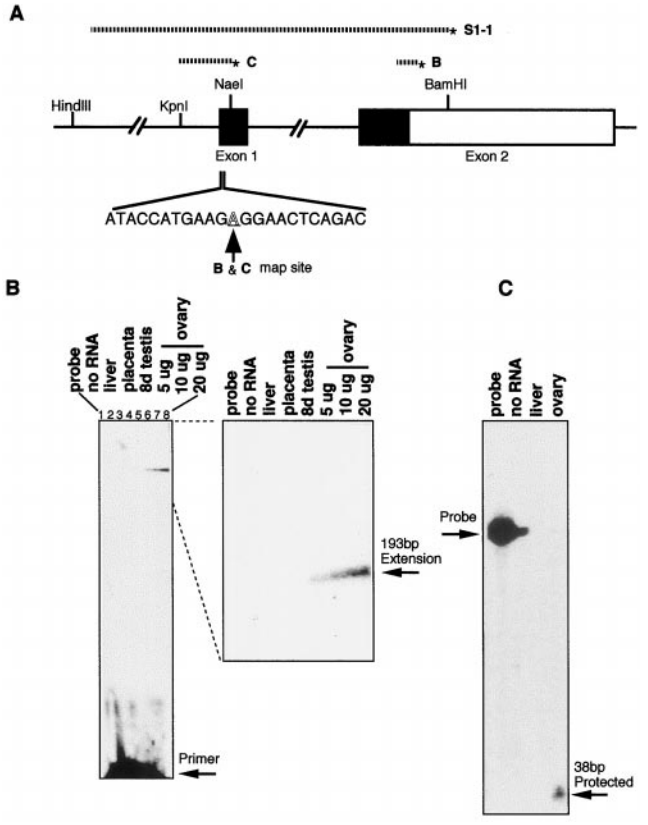


FIG. 2. Mapping of the transcriptional start site of the rat inhibin and activin $\beta_A$-subunit gene. A, Schematic diagram showing the 5′-end of the rat $\beta_A$-subunit gene and the locations of probes. Exons 1 and 2 are represented by *boxes*: *black box*, the nontranslated region defined by the identified start site; *open box*, translated $\beta_A$ precursor sequences. 5′-flanking sequences are shown by a *solid line*. The locations of the oligonucleotide primer (B) and S1 hybridization probes (S1–1, C) are shown by *broken lines*, with *asterisks* indicating the $^{32}$P-labeled ends. B and C, Autoradiograms of primer extension and S1 nuclease assays, respectively. The primer was hybridized with 5 $\mu$g poly (A)$^+$ RNA isolated from rat ovary or liver, placenta, 8d testis (negative controls). The probe was hybridized with 5 $\mu$g poly (A)$^+$ RNA isolated from rat ovary or liver (a negative control). *Arrows* in B and C mark the extended product and protected band, respectively.

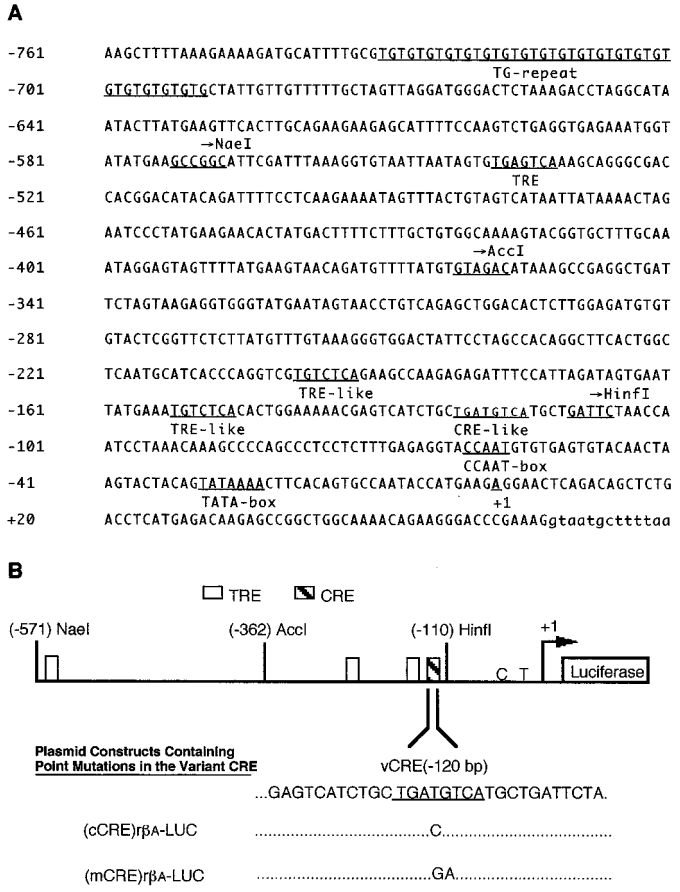


FIG. 3. Nucleotide sequence of the 5′ flanking region of the rat $\beta_A$-subunit gene and point mutations introduced into the CRE site. A, The DNA sequence of the promoter region is depicted and the transcriptional start site (+1) is indicated in *bold* and *underlined*. The restriction site end points used to generate 5′ deletion constructs are indicated by *arrows*, and the enzyme recognition site is *underlined*. The TATA and CCAAT boxes and sites related to cAMP/TPA regulation are indicated and *underlined*. A long (TG) repeat is *underlined* at position −700 bp. The variant CRE (TGATGTCA) is in *bold* and *underlined*. B, Schematic representation of the 5′-flanking region fused to the luciferase reporter gene. Indicated regulatory elements include the variant CRE at −120 bp, a TRE consensus sequence at −537 bp, and two variant TREs at −150 and −197 bp. The positions of the CCAAT and TATA boxes relative to the transcriptional start site are shown by C and T, respectively. The mutated constructs used in this study are also depicted. (cCRE)r$\beta_A$-Luc contains the palindromic consensus CRE in the context of the −571 bp promoter; (mCRE)r$\beta_A$-Luc contains a mutation in the core of the variant CRE converting TG→GA. The rat $\beta_A$-subunit promoter sequence has been deposited with GenBank (accession number AF045163).

promoter. Surprisingly, a similar decrease in activity was observed with the (cCRE)r$\beta_A$-Luc construct that has a consensus CRE. These results indicate that the variant CRE site in the rat inhibin and activin $\beta_A$ gene promoter is essential for mediating both FSK and TPA responses in GRMO2 cells. In addition, the finding that conversion to a consensus CRE impairs activity suggests that this element is not acting like a classical CREB-binding CRE.

### *The inhibin and activin $\beta_A$ variant CRE interacts with GRMO2 cell nuclear proteins*

To characterize the proteins that interact with the variant CRE, we performed electrophoretic mobility shift assays (EMSAs) using a double-stranded oligonucleotide corresponding to the activin $\beta_A$ promoter from −106 bp to −135 bp. Figure 6 shows formation of a predominant protein-DNA complex (lane 2) that is specifically competed by a 100-fold excess of the unlabeled wild-type $\beta_A$ variant CRE (lane 6) but not by a nonspecific oligonucleotide (lane 5). An oligonucleotide containing the mutant $\beta_A$ variant CRE partially competed the formation of this complex (lane 7), although much less effectively than the wild-type $\beta_A$ CRE. The sequence of the $\beta_A$ variant CRE is very similar to both CRE and TRE consensus sequences. We therefore used oligonucleotides containing CRE and TRE consensus sequences as competitors in an EMSA. As shown in lanes 3 and 4 of Fig. 6, both CRE and TRE consensus sequences abolished the binding of the protein complex to the $\beta_A$ variant CRE. These data indicate that the $\beta_A$ gene variant CRE protein-DNA complex may contain proteins of both the CREB/ATF and AP-1 fam-

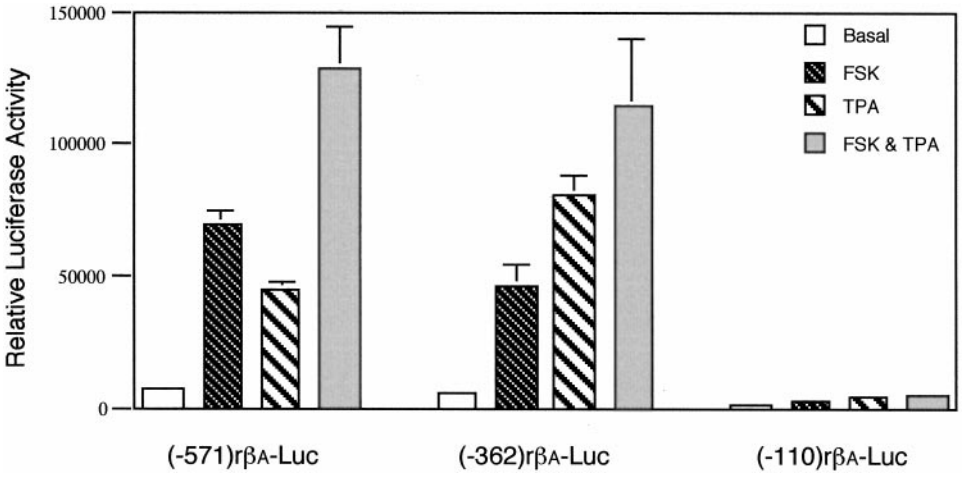


FIG. 4. The effects of FSK and TPA on rat $\beta_A$-subunit promoter activity. Presented are the results of transient transfections of GRMO2 cells with various r$\beta_A$-Luc 5′ deletion constructs. Relative luciferase activity was determined after normalization to total protein. A minimum of three independent experiments were carried out for each treatment group, and the results from a representative experiment are shown here. The SE is shown for treatment groups performed in triplicate.

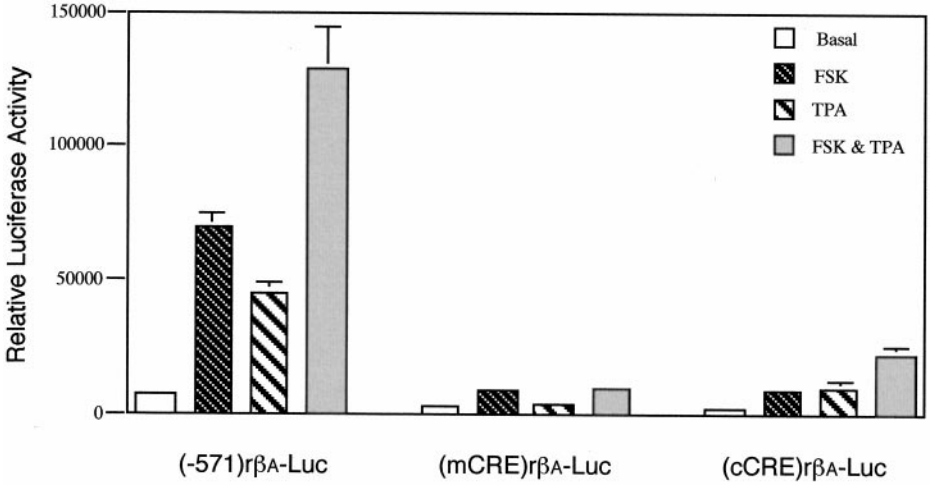


FIG. 5. The effects of variant CRE mutations on rat $\beta_A$-subunit promoter activity. Presented are the results of transient transfections of GRMO2 cells with various r$\beta_A$-Luc constructs. (cCRE) r$\beta_A$-Luc contains the palindromic consensus CRE and (mCRE)r$\beta_A$-Luc contains a mutation in the core of the variant CRE converting TG→GA. Relative luciferase activity was determined after normalization to total protein. A minimum of three independent experiments were carried out for each treatment group, and the results from a representative experiment are shown here. The SE is shown for treatment groups performed in triplicate.

ily of transcription factors, or at least includes proteins able to bind to either the CRE or TRE site.

*The protein complex binding the $\beta_A$ variant CRE contains members of the AP-1 family*

Having shown that a CRE or TRE can compete for factors bound to the $\beta_A$ gene variant CRE, we sought to determine if CREB/ATF or AP-1 family proteins were involved using antibodies to the respective factors, CREB, ATF-1, ATF-2 and jun-B, jun-D, c-jun, c-fos, fos-B, and fra-2 in EMSAs. Figure 7A shows that the CREB antibody did not affect the protein complex binding the variant CRE site (lane 3). Similarly, no significant interference with the protein complex was observed in the presence of the ATF-2 antibody, as shown in lane 5. However, in the presence of the ATF-1 antibody, the intensity of the protein-DNA complex is slightly reduced relative to the other bands (lane 4). Among the antibodies used to identify potential members of the AP-1 family in the protein complex binding the $\beta_A$ CRE, only the antibody to jun-B was observed to supershift the protein-DNA complex (lane 8), whereas the antibody to fos-B interfered with formation of the protein-DNA complex (lane 12) as shown in Fig. 7B. No significant change in formation of the protein complex on the $\beta_A$ CRE was observed in presence of antibodies to other members of the AP-1 family including, jun-D, c-jun, c-fos, fos-B, and fra-2 (lanes 9, 10, 11, and 13, respectively). Thus the variant CRE element appears to bind predominantly members of the AP-1 family of transcription factors, rather than CREB/ATF-1 family proteins, in GRMO2 cells.

*Activation of the rat $\beta_A$-subunit promoter by AP-1 family proteins in GRMO2 cells*

Based on the above gel mobility shift studies, jun-B and fos-B were analyzed for their ability to stimulate transcription of the rat $\beta_A$-subunit promoter in GRMO2 cells. Expression constructs for jun-B and fos-B were cotransfected with the reporter genes (−571)r$\beta_A$-Luc, (mCRE)r$\beta_A$-Luc, or (cCRE)r$\beta_A$-Luc into GRMO2 cells. As shown in Fig. 8, jun-B alone had little effect on promoter activity; however, fos-B significantly activated the −572-bp promoter. This activation was substantially higher when both jun-B and fos-B were coexpressed in GRMO2 cells. The (mCRE)r$\beta_A$-Luc was also activated by fos-B and jun-B, but to a lesser extent than the wild-type promoter, whereas the (cCRE)r$\beta_A$-Luc was not activated at all in response to fos-B/jun-B overexpression. These experiments indicate that the AP-1 family proteins

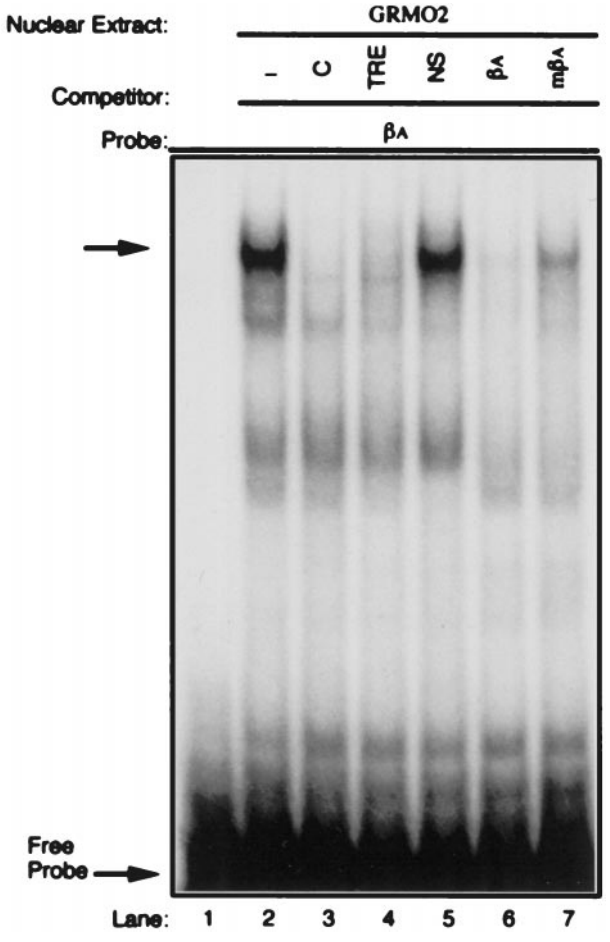


FIG. 6. Characterization of a protein complex binding the variant CRE in the rat $\beta_A$-subunit promoter. Presented is an EMSA using 5′ end-labeled double-stranded oligonucleotides containing the $\beta_A$ variant CRE ($\beta_A$) as probe with GRMO2 cell nuclear extracts. The GRMO2 cells were treated with FSK (10 $\mu$M) and TPA (30 nM) for 24 h before preparation of nuclear extracts. In each assay 10 $\mu$g of nuclear extract was incubated with 10,000 cpm of labeled probe for 20 min on ice and electrophoresed in a 5% nondenaturing acrylamide gel at 4 C for 2 h at 200 V. Some of the reactions included a 100-fold excess of unlabeled double-stranded oligonucleotides corresponding to the CRE consensus sequence (C), the AP-1 consensus sequence (TRE), the $\beta_A$-subunit vCRE ($\beta_A$), a mutant $\beta_A$-subunit vCRE (m$\beta_A$), or nonspecific DNA sequences (NS). Lane 1 is a control with probe with no nuclear extract. Lanes 2–7 show binding of the protein complex from GRMO2 cell nuclear extract to the labeled $\beta_A$ oligonucleotide in the presence or absence of the various competitor oligonucleotides. The *arrow* points to the specific DNA-protein complex observed.

jun-B and fos-B can transactivate the $\beta_A$-subunit gene through the variant CRE sequence.

## Discussion

The role of the pituitary gonadotropins in the regulation of the inhibin and activin subunit genes in granulosa cells has been well established using both animal and cell culture models (29). To investigate the molecular mechanisms underlying gonadotropin regulation of inhibin and activin gene expression, we have isolated and characterized the rat $\beta_A$-subunit gene. Our analyses indicate that the rat $\beta_A$-subunit gene consists of three exons. The genomic organization of the rat $\beta_A$-subunit gene reported here is structurally different from the rat inhibin and activin $\alpha$ and $\beta_B$-subunit genes, which contain only two exons (6, 16, 18, 30), in that the $\beta_A$ gene includes a small 5′-nontranslated exon. Using S1 nuclease mapping and primer extension, we have defined a unique transcriptional start site of the $\beta_A$-subunit gene, and have sequenced 761 bp of 5′ flanking DNA upstream of this start site. This led to the identification of potential binding sites for *trans*-acting factors known to mediate cAMP or phorbol ester-stimulated gene transcription. Three potential TRE or AP-1 binding sites were found between −140 bp and −450 bp in the inhibin and activin $\beta_A$-subunit promoter. A CRE-like sequence was found in the 5′-flanking region at −120 bp, which is similar in position to the cAMP-responsive element in the promoter of the rat $\alpha$ inhibin gene (16). A track of TG repeats was found to extend from −691 bp to −732 bp of the $\beta_A$ promoter. We previously reported that a similar TG repeat found in the rat $\alpha$ inhibin gene affected basal promoter activity but did not affect FSK-responsiveness (16). We have not yet tested the role of the TG repeat in $\beta_A$-subunit gene expression.

Recently, the genomic structure of the human inhibin and activin $\beta_A$-subunit gene has been reported (26), and comparison of the 5′-flanking regions to −275 bp of the two genes shows a greater than 90% sequence identity. Furthermore, the human $\beta_A$ gene promoter has also been shown to be activated in response to 8-Br-cAMP and TPA (26). The strong conservation of potential transcription factor binding sites, including the TRE and variant CRE sites discussed here, in the 5′-flanking regions of the rat and human $\beta_A$-subunit genes suggests that these sites are likely to play an important role in the regulation of $\beta_A$-subunit gene expression.

The expression patterns of the $\alpha$-, $\beta_A$-, and $\beta_B$-subunit genes in granulosa cells during the estrous cycle (11) and in response to a variety of other hormones, growth factors and steroids (10, 29) have been well documented. However, very little is known about the signaling pathways and the intracellular effector molecules regulating the expression of these genes in response to physiological signals that change rapidly during the reproductive cycle. This has been due in part to the unavailability of appropriate granulosa cell lines. Recently, a mouse granulosa cell line designated GRMO2 was generated (21) that exhibits many features characteristic of primary granulosa cells in culture, including the expression and secretion of inhibin and activin (31). In this study, we found that GRMO2 cells express the transfected $\beta_A$-subunit gene promoter and regulate its activity in response to cAMP and TPA treatment. While the studies reported here use the GRMO2 granulosa cell line, we have found the $\beta_A$-subunit gene promoter to be similarly regulated by cAMP in transfected primary rat granulosa cells (Romanelli, J. C. D., and K. E. Mayo, unpublished results).

The major signaling pathway mediating the regulation of the inhibin and activin subunit genes by pituitary gonadotropins in ovarian granulosa cells is likely to be the cAMP-PKA pathway (29). We reported previously that a CRE-like element in the rat inhibin $\alpha$-subunit gene promoter mediates the cAMP response in primary granulosa cells (16). In the present study, we have shown that the variant CRE in the $\beta_A$-subunit gene is capable of mediating cAMP induction of

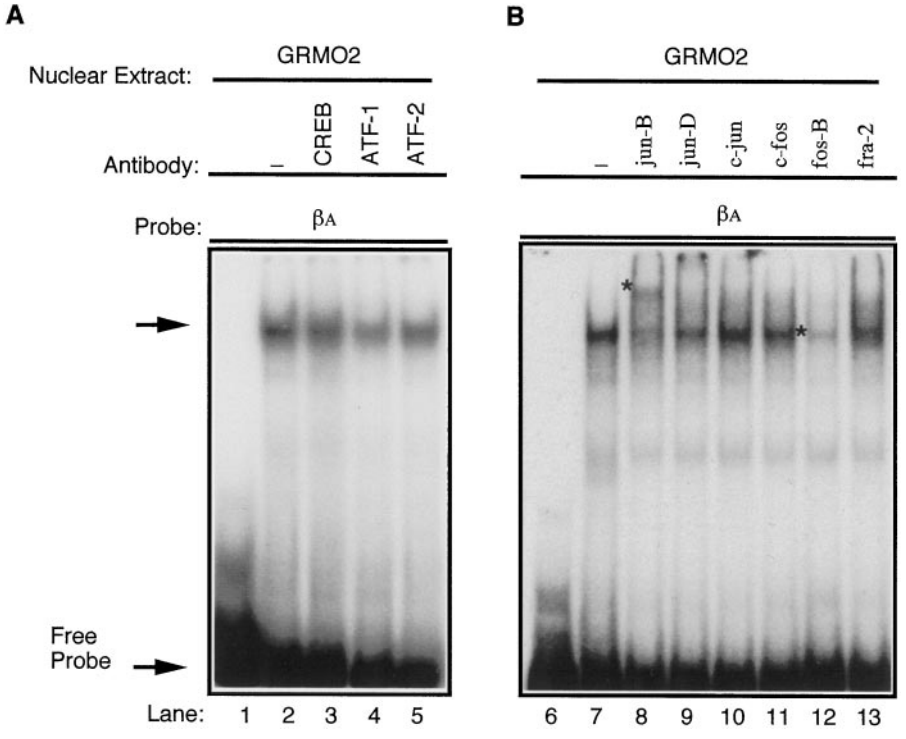


FIG. 7. Identification of nuclear proteins binding to the variant CRE in the rat $\beta_A$-subunit promoter. Presented is an EMSA using labeled double-stranded 30-bp $\beta_A$ variant CRE ($\beta_A$) oligonucleotide as probe with GRMO2 cell nuclear extract in the presence of antibodies recognizing proteins in the CREB/ATF family (A) or AP-1 family (B). GRMO2 nuclear extract (10 $\mu$g) was incubated with the appropriate antibodies for 45 min at room temperature before the addition of labeled $\beta_A$ probe ($10^4$ cpm) for 20 min on ice. All reaction mixtures were electrophoresed in a 5% nondenaturing acrylamide gel at 4 C for 2 h at 200 V. A, Lane 1, Free probe. Lanes 2–5, GRMO2 nuclear proteins binding to the variant CRE in the abscence of antibody or the presence of antibodies against CREB, ATF-1 and ATF-2, as indicated. B, Lane 6, free probe. Lanes 8–13, GRMO2 nuclear proteins binding to the variant CRE in the abscence of antibody or the presence of antibodies against the indicated AP-1 family proteins. The *asterisks* show the shifted band (lane 8) or the reduced level of protein binding (lane 12) to the variant CRE.

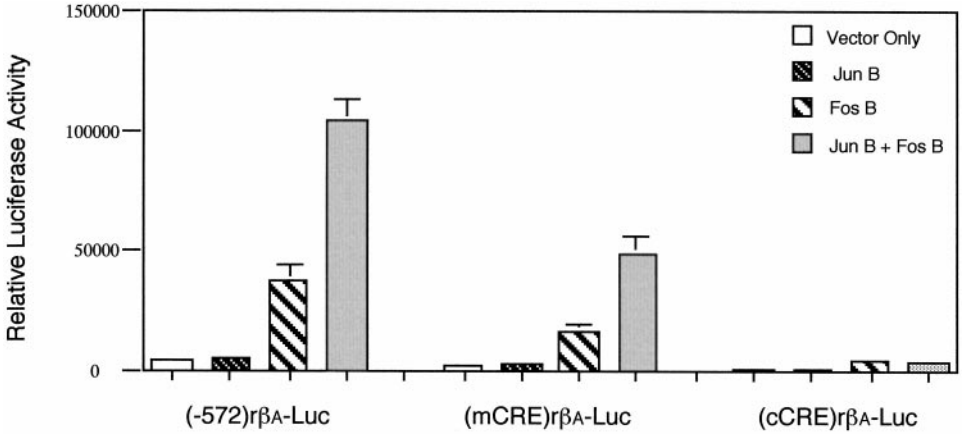


FIG. 8. Effect of overexpression of jun B and fos B on the activity of the rat $\beta_A$-subunit promoter in GRMO2 granulosa cells. Expression constructs for the AP-1 family proteins jun-B and fos-B were cotransfected with the reporter genes (−572)r$\beta_A$-Luc, (cCRE) r$\beta_A$-Luc, and (mCRE)r$\beta_A$-Luc into GRMO2 cells. Relative luciferase activity was determined 48 h later after normalization to total protein. A minimum of three independent experiments were carried out for each treatment group, and the results from a representative experiment are shown here. The SE is shown for treatment groups performed in triplicate.

transcription in GRMO2 granulosa cells. Although cAMP regulation was first associated with the presence of a palindromic consensus CRE in gene promoters, in the past few years it has become increasingly evident that nonconsensus CREs are also able to mediate PKA responsiveness in a number of genes (32–36). In addition, the flanking sequence context of CRE and CRE-related elements is reported to be important for their activity (37, 38). It has been suggested that the weaker contribution of a variant CRE can be compensated for by the activity of sequences surrounding the CRE (37). Our results indicate that the conversion of the variant CRE to a consensus CRE does not enhance promoter activity, as has been reported to occur with the glycoprotein hormone $\alpha$-subunit gene promoter in a trophoblast cell line (39). In addition, the $\beta_A$ variant CRE was found to be responsive to TPA, an agent known to activate the PKC signaling pathway and induce effector molecules that classically act through a TRE consensus sequence, TGAC/GTCA. Taken together, our findings suggest that the unique sequence of the variant CRE found in the rat $\beta_A$-subunit gene promoter is essential for regulation of the gene and that this variant CRE does not function as a classical CREB-binding element.

The protein kinase A and protein kinase C signaling pathways appear to be major regulators of $\beta_A$-subunit gene expression in a variety of cell types. An elevation of $\beta_A$ mRNA species in human fibrosarcoma HT 1080 cell lines in response to 8-bromo-cAMP and TPA has been reported (27, 40). In human adrenal cells, ACTH, cAMP, and TPA stimulate $\beta_A$-subunit transcripts (15). Recently, $\alpha$ and $\beta_A$ mRNA levels were shown to be induced by 8-bromo-cAMP and TPA in human granulosa-luteal cells in culture (28). The data presented here demonstrate that the variant CRE in the promoter of the $\beta_A$-subunit gene is likely to mediate induction by both these signaling pathways. Although CRE-like elements have been shown to mediate responsiveness to the PKC signaling pathway in other genes (34, 35), the ability of

the $\beta_A$-subunit gene variant CRE to respond strongly to both signaling pathways appears to be unique. In cultured rat granulosa cells, stimulation of the PKA or PKC signaling pathways has been demonstrated to differentially favor the formation of inhibin or activin, respectively (41). Recently the $\alpha$- and $\beta_A$-subunit genes in human granulosa-luteal cells were reported to be differentially expressed in response to treatment with cAMP or phorbol esters (28), in that the cAMP was found to have a predominant effect on $\alpha$-subunit expression, whereas TPA was a potent stimulator of $\beta_A$-subunit gene expression but did not affect $\alpha$-subunit mRNA levels. We have made similar observations in rat granulosa cells (Pei, L., and K. E. Mayo, unpublished results). Taken together, these observations indicate that the level of induction of the $\alpha$- and $\beta_A$-subunit genes in response to these two signaling pathways may be critical regulatory step for the formation of inhibins *vs.* activins in the granulosa cell.

The regulation of gene expression by the PKA and PKC signaling pathways is often mediated by two related regulatory elements, the CRE (TGACGTCA) and TRE (TGAC/GTCA), respectively (42, 43). Generally, CRE and TRE elements are thought to bind distinct sets of transcription factors, the CREB/ATF and Fos/Jun family of proteins, respectively, both of which belong to the bZip (basic region leucine-zipper) family of proteins (43). However, an interaction between these two inducing pathways at the transcriptional level is an increasingly common finding. For example, AP-1 family proteins can bind a CRE site (44, 45), and the nuclear factors CREB and CREM can bind to AP-1 sites (46). Although cAMP and TPA are classical inducers of the PKA and PKC signaling pathways, respectively, cross-talk between these two pathways has been reported. For example, FSH (an inducer of the PKA pathway) is reported to stimulate Jun-B and c-Fos expression in rat Sertoli cells (47), and hCG stimulates c-Fos expression in rat ovarian granulosa cells (48). Conversely, phorbol esters (TPA) have been shown to induce adenylate cyclase phosphorylation (49), affecting production of the cAMP second messenger, and to stimulate CREB phosphorylation (50).

We reported previously that the $\beta_A$-subunit variant CRE binds recombinant CREB protein, as established using DNA footprinting analysis (51). However, in comparison with a consensus CRE, the binding affinity is substantially reduced, consistent with strict sequence requirements for high affinity CREB binding (38). Recently, a variant CRE in the promoter of the glycoprotein hormone $\alpha$-subunit gene, identical in sequence to the variant CRE in the promoter of the $\beta_A$-subunit gene, has been shown to bind a protein complex that cross-reacts with antibodies against AP-1 family transcription factors in gonadotrope and trophoblast cells (37). Based on our results, it appears that mediation of the effects of the PKA and/or PKC signaling pathways in GRMO2 granulosa cells occurs predominantly through AP-1 family proteins. However, we cannot rule out the possibility of hetero-dimerization between members of the CREB/ATF and AP-1 family, which has been previously reported (52), and it will be important to establish whether such heterodimeric protein complexes form on the $\beta_A$-subunit variant CRE in response to stimulants that can activate both the PKA and PKC signaling pathways in granulosa cells.

In summary, our results demonstrate a direct transcriptional regulation of the inhibin and activin $\beta_A$-subunit gene in response to cAMP and TPA, show that a variant CRE in the promoter of the $\beta_A$-subunit gene is critical for this regulation, and identify AP-1 family proteins as playing an important role in mediating this regulation. It will be important in future studies to analyze in greater detail the sequence requirements for activity of this novel CRE-like element, to establish more completely the repertoire of proteins able to mediate regulation through this element, and to investigate ways in which signals from the PKA and PKC pathways are integrated to appropriately regulate inhibin and activin subunit gene expression in the ovarian granulosa cell.

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