

GnRH Regulates Early Growth Response Protein 1 Transcription Through Multiple Promoter Elements

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Pulsatile secretion of GnRH is the major regulator of gonadotropin (LH, FSH) gene expression and secretion. Recently, GnRH has been shown to rapidly stimulate the expression of early growth response protein-1 (Egr-1), a transcription factor that is essential for LH β gene expression in the pituitary. In this study, we examined the regulatory elements and signal transduction pathways by which GnRH regulates Egr-1 transcription. Deletion analysis of the murine Egr-1 promoter identified two regions (-370 to -342 and -116 to -73) that are critical for GnRH responsiveness in α T3 pituitary gonadotrope cells. The first region, which contains two serum response elements (SREs), contributed about 70–80% of GnRH inducibility, whereas the second region, which contains two SREs and one Ets binding site, conferred

an additional 20–30% of activity. Mutations that abolish protein binding to these SREs and Ets binding sites completely eliminated GnRH-mediated transcriptional activation of the Egr-1 promoter. Mutation of cAMP response element reduced promoter activity by 40%. Using specific protein kinase inhibitors, GnRH stimulation of Egr-1 expression was found to be dependent on PKC/ERK pathways. In addition, GnRH activated p90 ribosomal S6 kinase, which has the potential to phosphorylate serum response factor and cAMP response element binding protein. We conclude that GnRH stimulation of Egr-1 gene expression requires several distinct SREs/Ets elements and a cAMP response element and is mediated via activation of PKC/ERK signaling pathways. (*Molecular Endocrinology* 16: 221–233, 2002)

GNRH PLAYS AN essential role in the regulation of gonadotropin gene expression and secretion (1–3). The gonadotropins (LH and FSH) consist of a common α -subunit and specific β -subunits. The α - and β -subunits are each stimulated by GnRH at the transcriptional level (4, 5). A pulsatile GnRH stimulus is required to stimulate gonadotropin subunit transcription and the α , LH β , and FSH β genes are differentially regulated by pulse frequency (4, 6). For instance, LH β gene expression is maintained by relatively high-frequency GnRH pulses, whereas FSH β expression is favored by lower pulse frequencies (4).

Studies of the α -subunit gene promoter have identified several transcription factors and regulatory elements that provide basal, tissue-specific, and hormonally mediated regulation of gene expression (7–10). Three transcription factors—the orphan nuclear receptor steroidogenic factor 1 (Sf-1), the *bicoid*-related homeoprotein Ptx1, and the early growth response protein-1 (Egr-1)—are essential for LH β transcriptional activation (11–16). Among these factors, Egr-1 appears to be a dynamic effector of GnRH ac-

tion. GnRH stimulates Egr-1 expression but has little or no effect on the levels of Ptx1 or Sf-1 (13, 15).

The Egr-1 gene (also referred as *krox24*, *NGFI-A*, *TIS8*, or *zif268*) belongs to a family of immediate early response genes (17, 18). The Egr family of proteins contain a conserved zinc finger DNA-binding domain and bind to a GC-rich sequence in the promoter region of target genes (17). Egr-1 expression is rapidly and transiently activated in many different cell types during development. In adult tissues, a variety of signals, including serum, growth factors, cytokines, and hormones, stimulate Egr-1 expression (17). Egr-1 is also induced by changes in the local cellular environment, such as variation in osmotic pressure, heat shock, hypoxia, and DNA-damaging agents (19–23). Egr-1 is involved in diverse cellular functions including cell proliferation, differentiation, and apoptosis (17). A role for Egr-1 in reproduction was demonstrated in two independent Egr-1 gene deletion experiments (11, 12). Egr-1-deficient mice are sterile due to defects in the anterior pituitary of both sexes and in the ovaries of females (12). In the pituitary, the absence of Egr-1 causes a selective loss of LH β synthesis and secretion whereas production of the α -subunit and FSH β remain intact (12). Sequence analysis of the rat LH β gene promoter identified two Egr-1 binding sites (11, 14). Egr-1 binds to both sites and activates the LH β promoter by acting in combination with Sf-1 (11, 14).

The 5'-flanking sequence of the Egr-1 gene has been cloned from several species (24–28), revealing

Abbreviations: CBP, CREB-binding protein; CREB, cAMP response element-binding protein; DMSO, dimethylsulfoxide; Egr-1, early growth response protein 1; GM-CSF, granulocyte macrophage colony-stimulating factor; JNK, Jun N-terminal kinase; p90RSK, p90 ribosomal S6 kinase; PMA, 12-myristate 13-acetate; SAPK, stress-activated protein kinase; Sf-1, steroidogenic factor 1; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor.

the presence of multiple serum response elements (SREs). Additional putative regulatory elements include an Sp1 binding site, a cAMP response element (CRE), and an Ets binding site (17, 26, 29). The majority of studies have mapped Egr-1 promoter inducibility to regions encompassing the SRE clusters, although preferential utilization of specific SREs may occur in response to specific stimulatory pathways (30-34). The SRE consensus sequence contains a CC(A/T)₆GG motif, and activation of the SRE is dependent on binding of the transcription factor, SRF (serum response factor) (27, 35, 36). Ets binding sites are located adjacent to the SREs of the Egr-1 promoter and thereby permit the interaction of ternary complex factors (TCFs) with the SRE-SRF complex (37, 38). Studies showed that SRF forms a ternary complex with a TCF protein, Elk-1, at the *c-fos* SRE, and phosphorylation of Elk-1 is required for *in vitro* ternary complex formation with SRF (39, 40). Like the *c-fos* SRE, the Egr-1 SRE/Ets sites are occupied by a multiprotein complex containing a dimer of SRF and Ets family transcription factors (30). In addition to the SREs and Ets motif, a proximal CRE is required for stimulation by granulocyte-macrophage colony-stimulating factor (GM-CSF) (32, 41), whereas a distal CRE is necessary for activation by p38/stress-activated protein kinases (SAPKs) in response to cellular stress (20). The CRE plays a positive role in Egr-1 expression in response to depolarization in pancreatic islet β -cells (42) but serves as a negative regulator in synovial fibroblasts from rheumatoid arthritis patients (43).

Activation of the Egr-1 promoter is mediated by several kinase pathways. A member of the MAPK family, p38/SAPK2 (or p38MAPK), is involved in stress-induced stimulation of Egr-1 (20), whereas PKC, and ERK-1 and -2, are responsible for the Elk phosphorylation, which ultimately mediates Egr-1 induction (19, 44, 45). Activation of the serine/threonine kinase, p90RSK (p90 ribosomal S6 kinase), which phosphorylates cAMP response element binding protein (CREB), is critical for Egr-1 stimulation in response to GM-CSF (34, 46).

Although GnRH is known to transiently induce Egr-1 expression in gonadotrope cells (13, 15, 16, 47, 48), the mechanisms and intracellular signaling pathways by which GnRH regulates Egr-1 expression remain incompletely defined. In the present study, we investigated the 5'-regulatory elements of the murine Egr-1 gene in cultured gonadotrope cells to identify GnRH-mediated signaling events and transcription factors that stimulate the Egr-1 promoter.

RESULTS

GnRH Activates the Egr-1 Promoter Through SREs and Adjacent Ets Binding Sites

To define the regulatory elements responsible for GnRH stimulation, a 1.3-kb fragment of the murine

Egr-1 promoter was sequentially deleted. As shown in Fig. 1, the -1,381 reporter construct displayed a 6- to 7-fold increase in response to GnRH when transiently transfected into α T3 cells. The fold induction by GnRH was relatively constant until deletions reached -370 bp. Further deletion resulted in a significant loss of activity. Approximate 2-fold stimulation by GnRH was maintained up to -116 bp. Based on these findings, key elements that confer GnRH responsiveness appear to be located in two regions (-370 to -342 and -116 to -73). The distal region (region A) includes two SREs, and the proximal region (region B) contains two SREs on both sides of an Ets binding site (Fig. 2A).

SRF Binds to Both Distal and Proximal SRE Sites in the Egr-1 Promoter

EMSA were performed to document specific transcription factor binding to the SRE and Ets sites. Nuclear extracts prepared from α T3 cells were incubated with radiolabeled oligonucleotide probes corresponding to regions A and B of the Egr-1 promoter (Fig. 2A). A single complex was observed with the region A probe (Fig. 2B, lane 1). This complex was eliminated by mutations in the two SREs within region A (Fig. 2B, lane 2) or by a 100-fold excess of unlabeled oligonucleotides (Fig. 2B, lane 3). An anti-SRF antibody supershifted the complex completely (Fig. 2B, lane 4), suggesting that SRF is the primary gonadotrope-derived transcription factor that binds to the distal SREs. Two complexes were detected using the region B probe, which contains two SREs and an Ets binding site (Fig. 2B, lane 5). Both DNA-protein complexes were eliminated by mutations of the SRE/Ets binding

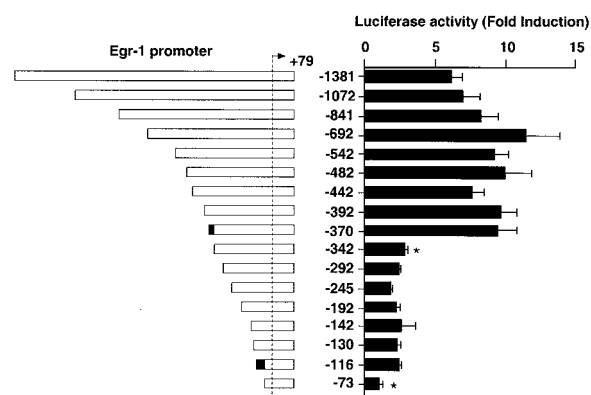


Fig. 1. Localization of Regions Involved in GnRH Stimulation of the Murine Egr-1 Promoter

A deletion series of the Egr-1 promoter-reporter constructs was transiently transfected into α T3 cells (0.5 μ g/well), and cells were treated with or without 10 nM GnRH for 4-6 h before luciferase assays. Results are the mean \pm SEM of fold induction. Data represent at least three independent experiments, with each transfection performed in triplicate. Two critical regions conferring GnRH responsiveness are shown in shaded areas. *, $P < 0.05$ for construct -342 vs. -370 and construct -73 vs. -116.

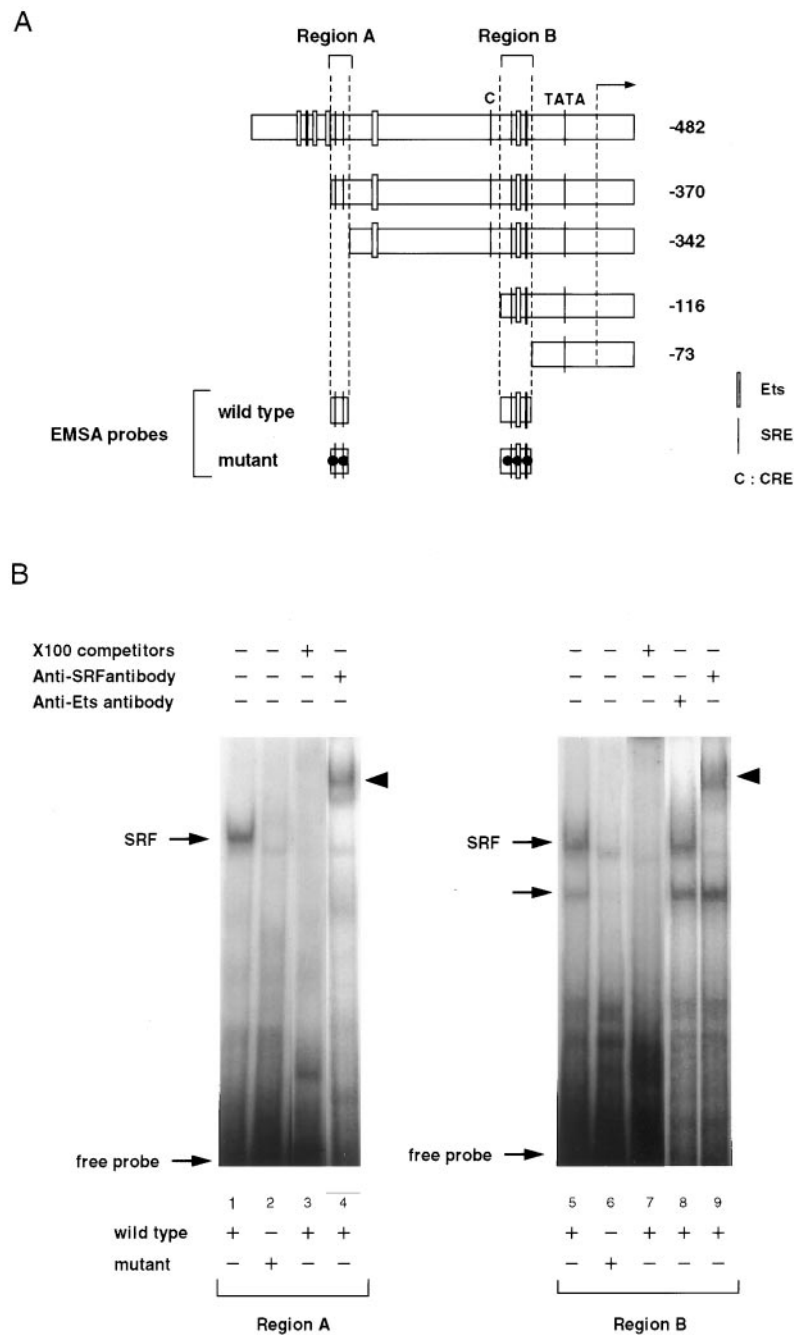


Fig. 2. Protein Binding to Wild-Type or Mutant SREs and Ets Motifs in the Egr-1 Promoter

A, Schematic representation of the putative regulatory elements within the Egr-1 promoter. Region A contains two SREs, and region B contains two SREs and an Ets binding site. Mutations introduced to the EMSA probes are also shown. B, Nuclear extracts were prepared from α T3 cells. EMSA was performed with wild-type or mutant radiolabeled oligonucleotides.

site (Fig. 2B, lane 6) or by addition of excess unlabeled oligonucleotides (Fig. 2B, lane 7). Incubation with an anti-Ets antibody did not supershift either band (Fig. 2B, lane 8). However, anti-SRF antibody completely supershifted the upper complex (Fig. 2B, lane 9), indicating that SRF is also bound to the proximal SRE. The binding activity of SRF and Ets was not affected by treatment of the cells with GnRH (data not shown).

Mutations in the Egr-1 SREs Impair Transcriptional Activation by GnRH

Mutations that eliminated protein binding to regions A and B (Fig. 2A) were introduced into the -370 construct (-370 m) to assess the functional roles of these binding sites. The native -370 construct was stimulated 8-fold by GnRH treatment, whereas the -370 m

construct failed to respond to GnRH (Fig. 3), indicating an essential contribution of the distal and proximal SREs to GnRH stimulation of the Egr-1 promoter.

CREB Is Associated with the CRE Egr-1 Promoter

Because the Egr-1 promoter CRE has been shown to mediate induction in response to certain cytokines (32) and stress (20), it is possible that the CRE is also involved in GnRH regulation. Oligonucleotide probes containing the CRE (TCACGTCA) from the Egr-1 promoter were examined in EMSA using human embryonic kidney TSA 201 cell nuclear extracts (Fig. 4A). Two binding complexes were observed with the Egr-1 promoter CRE (Fig. 4B, lane 1). The intensity of the upper band slightly decreased when an anti-CREB antibody was added compared with the control with normal serum (Fig. 4B, lanes 2 and 3). When TSA cells were transfected with the CREB expression vector, the intensity of the upper band increased dramatically (lanes 4–6), indicating that CREB is a component of this complex. Incubation with an anti-CREB antibody resulted in a supershifted band (Fig. 4B, lane 5). Similar results were obtained with an oligonucleotide derived from the CRE sequence of the inhibin α promoter (49) (Fig. 4B, lanes 7–12), although the lower band was not detected with the inhibin α promoter CRE. To examine the effect of GnRH on CREB binding to the Egr-1 promoter CRE, α T3 cells were treated with or without GnRH. Similar to TSA cells, two DNA-protein complexes bound to the wild-type CRE (Fig. 4B, lane 13), but they were not observed with the mutant CRE (TCICATCA) probe and were eliminated by excess unlabeled oligonucleotides (Fig. 4B, lanes 14 and 15). The intensity of the faster-migrating band was not affected by an anti-CREB antibody. This band was present in both TSA and α T3 cells and appeared to be Egr-1 promoter specific, as it was not detected with

the inhibin α promoter CRE. Addition of the anti-CREB antibody decreased the signal of the slower-migrating band slightly and generated faint supershifted bands, but the intensity of these bands was not altered by GnRH treatment (Fig. 4B, lanes 16 and 17). EMSA was also performed using an antiphospho-CREB antibody that specifically recognizes protein phosphorylated on Ser 133. In the absence of GnRH, a distinct supershifted band was observed, and GnRH treatment significantly increased the intensity of this band (Fig. 4B, lanes 18 and 19).

The Egr-1 CRE Contributes to GnRH Induction of the Egr-1 Promoter

The CRE in the -692 reporter construct was mutated (-692 m) to examine the functional role of the CRE. The native -692 construct was stimulated 6- to 7-fold after GnRH treatment (Fig. 5). Introduction of the CRE mutation resulted in a 40% loss of activity in response to GnRH. Basal reporter activity was not affected by the mutation.

PKC and ERK Pathways Are Involved in GnRH Stimulation of the Egr-1 Promoter

Multiple intracellular pathways contribute to Egr-1 expression (19, 20, 44, 46, 50, 51). To elucidate the individual contribution of intracellular signaling pathways to GnRH regulation of Egr-1 gene expression, α T3 cells were transfected with the -442 reporter construct and treated with or without GnRH in the presence or absence of specific protein kinase inhibitors. As shown in Fig. 6, GF109203X completely blocked transcriptional activation of the Egr-1 promoter by GnRH but had no effect on basal activity. Pretreatment with 12-myristate 13-acetate (PMA) to deplete PKC abolished both GnRH and PMA-induced Egr-1 promoter activity (data not shown). Likewise, the ERK inhibitor, PD98059, totally blocked GnRH stimulation of the Egr-1 promoter and exhibited a slight inhibitory effect on basal activity (Fig. 6). KN62, a Ca^{2+} /calmodulin-dependent kinase inhibitor, caused a slight decrease in the promoter activity by GnRH. A PKA inhibitor, H89, showed a partial inhibition of the Egr-1 promoter by GnRH.

Western blot analyses showed that Egr-1 protein is strongly induced by 1-h treatment of α T3 cells with GnRH (Fig. 7A). In agreement with the transfection data, treatment with GF109203X or PD98059 substantially blocked GnRH stimulation of Egr-1 protein expression. KN62 and H89 exhibited weak inhibitory effects (Fig. 7, B and C).

Dose-response experiments were performed to further evaluate the effects of GF109203X on GnRH-induced Egr-1 promoter activity. PMA and forskolin were included as positive and negative controls, respectively. PMA stimulation of Egr-1 promoter activity was reduced by 50% using $0.1 \mu\text{M}$ GF109203X. The stimulatory effect of GnRH was less affected (17% inhibition) at this GF109203X concentration (Fig. 8). Using $0.5 \mu\text{M}$ or

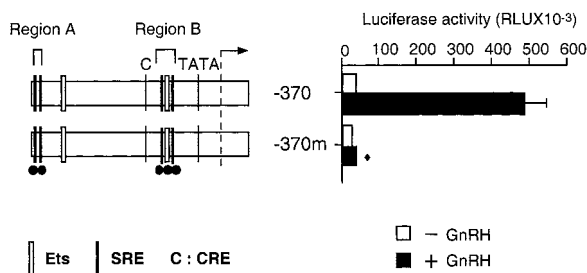


Fig. 3. Effect of Mutations of the SREs and Ets Sites on GnRH Inducibility of the Egr-1 Promoter

The wild-type -370 reporter construct and constructs containing mutations at SREs and Ets binding sites within regions A and B (-370 m) were transiently transfected into α T3 cells ($0.5 \mu\text{g}/\text{well}$). After treatment with or without 10 nM GnRH for 4–6 h, cells were harvested and luciferase activity assays were performed. Values shown are the mean \pm SEM. Data represent three experiments, with each transfection performed in triplicate. † , $P > 0.05$ vs. basal activity.

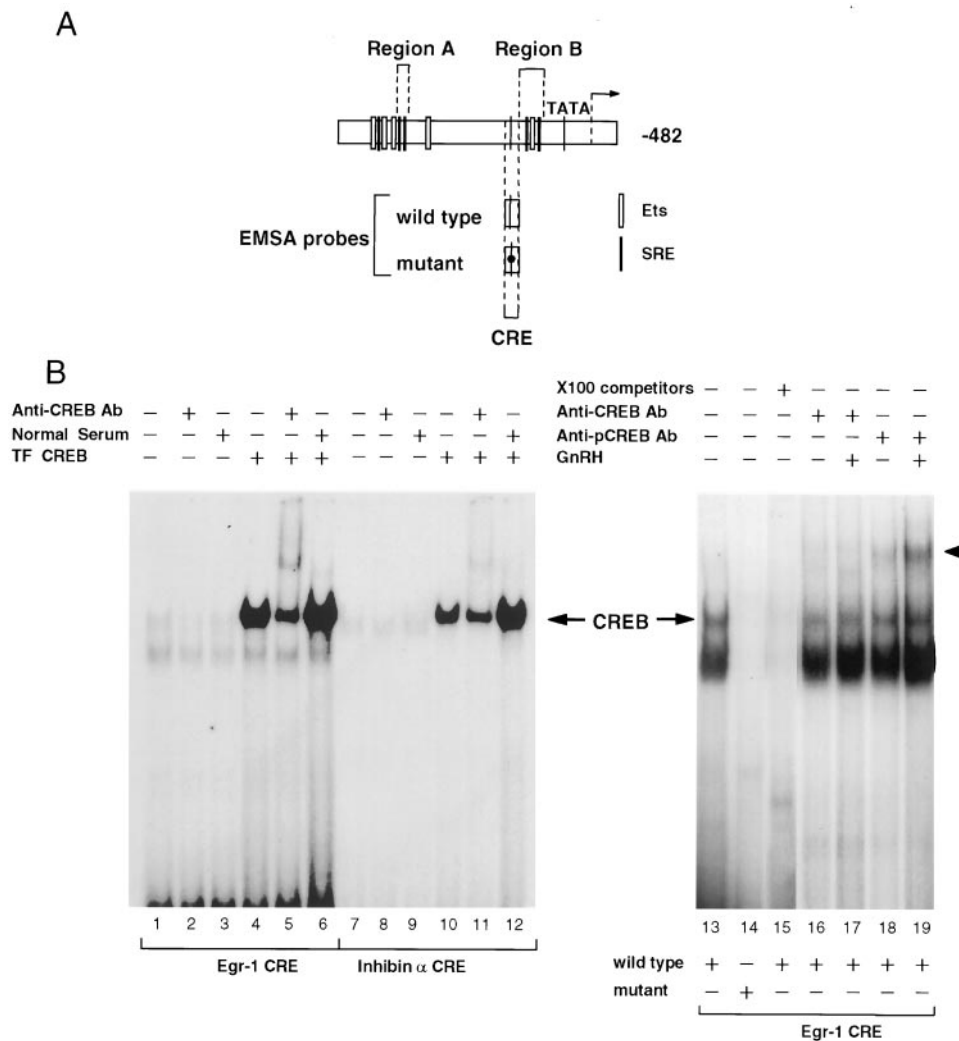


Fig. 4. CREB Bound to the CRE in the Egr-1 Promoter

A, Schematic representation of the CRE within the Egr-1 promoter. The CRE is located between regions A and B. Mutations introduced into the EMSA probe are also shown. B, Nuclear extracts were prepared from TSA cells transfected with a control or CREB expression vector (TF CREB) and α T3 cells treated with or without 10 nM GnRH for 15 min, and EMSA was performed using wild-type or mutant Egr-1 CRE probes and the inhibin α CRE. The arrowhead indicates the supershifted band.

greater (1, 5, and 10 μ M) GF109203X concentrations, both GnRH and PMA-induced activation of the Egr-1 promoter were completely blocked (Fig. 8). As expected, forskolin did not alter Egr-1 promoter activity.

GnRH Induces Activation of p90RSK

p90RSK, an immediate downstream kinase of MAPK pathway, is activated in pokeweed mitogen-treated B cells (52) and in differentiated monocytes (34) in association with Egr-1 induction. Recent evidence indicates that p90RSK can directly phosphorylate SRF on Ser-103 *in vitro* (53) and is responsible for CREB phosphorylation at Ser-133 in response to growth factor stimulation (46, 54–56). We next examined whether GnRH stimulates p90RSK activation using an antibody that recognizes phosphorylated p90RSK. α T3 cells were treated with

GnRH in the absence or presence of protein kinase inhibitors. Immunoblot analysis revealed a 3- to 4-fold increase in p90RSK phosphorylation after GnRH stimulation (Fig. 9A). As a positive control, PMA also stimulated p90RSK phosphorylation. The activation of p90RSK by GnRH and PMA was partially blocked by 1 μ M GF109203X (Fig. 9A). Higher concentrations of GF109203X (10 μ M) inhibited GnRH activation of p90RSK by 90% (data not shown). PD98059 totally prevented the p90RSK phosphorylation by GnRH (Fig. 9A). Forskolin had no effect on p90RSK activation.

GnRH Stimulates Elk-1 Phosphorylation Through a PKC-Independent ERK Pathway

To explore a link between MAPK and the SRE-associated transcription factors in response to GnRH stimula-

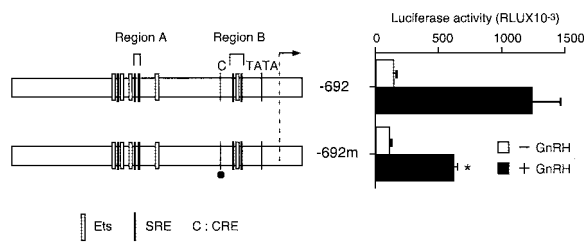


Fig. 5. Effect of CRE Mutations on GnRH Stimulation of the Egr-1 Promoter

A mutation was introduced into the CRE of the -692 reporter gene ($-692m$). Wild-type or mutant reporter constructs were transiently transfected into $\alpha T3$ cells ($0.5 \mu\text{g}/\text{well}$). Cells were treated with or without 10 nM GnRH for 4–6 h, followed by luciferase assays. Values shown are the mean \pm SEM. Data represent three independent experiments, with each transfection performed in triplicate. *, $P < 0.05$ for $-692m$ vs. -692 in GnRH-treated cells.

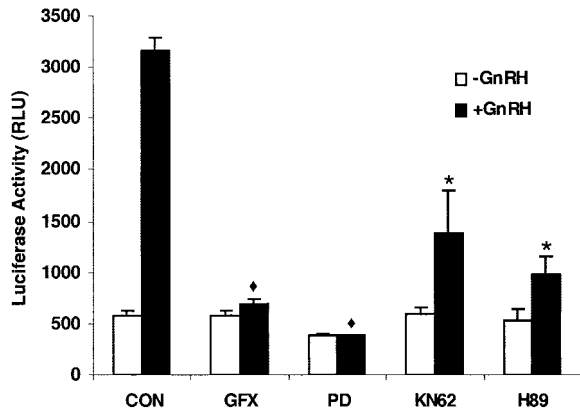


Fig. 6. Effects of Protein Kinase Inhibitors on the Egr-1 Promoter Activity

$\alpha T3$ cells were transiently transfected with the -442 -bp Egr-1 promoter construct ($0.5 \mu\text{g}/\text{well}$). Forty hours after the transfection, cells were preincubated with dimethylsulfoxide [DMSO (control)], GF109203X ($5 \mu\text{M}$), PD98059 ($50 \mu\text{M}$), KN-62 ($5 \mu\text{M}$), or H-89 ($10 \mu\text{M}$) for 1 h. Cells were then treated with or without 10 nM GnRH for 4–6 h (+, $P > 0.05$ vs. basal activity. *, $P < 0.05$ vs. GnRH-treated cells of control group). Cell extracts were prepared and assayed for luciferase activity. Values shown are the mean \pm SEM. Data represent four independent experiments, with each transfection performed in triplicate.

tion, we determined whether GnRH stimulates Elk-1 activation and which signaling pathways are involved. Studies of the *c-fos* SRE showed that SRF forms a ternary complex with an accessory protein, Elk-1, which is essential for *c-fos* stimulation (37, 57). Elk-1 is a well documented major nuclear substrate of MAPK cascades (38, 40, 58), and Elk-1 phosphorylation contributes to SRE-mediated transcriptional activation (45, 57, 59–62). As shown in Fig. 9B, GnRH increased Elk-1 phosphorylation in $\alpha T3$ cells. This stimulatory effect of GnRH was not affected by $1 \mu\text{M}$ GF109203X or by higher concentrations of GF109203X (5 and $10 \mu\text{M}$, data not shown). In

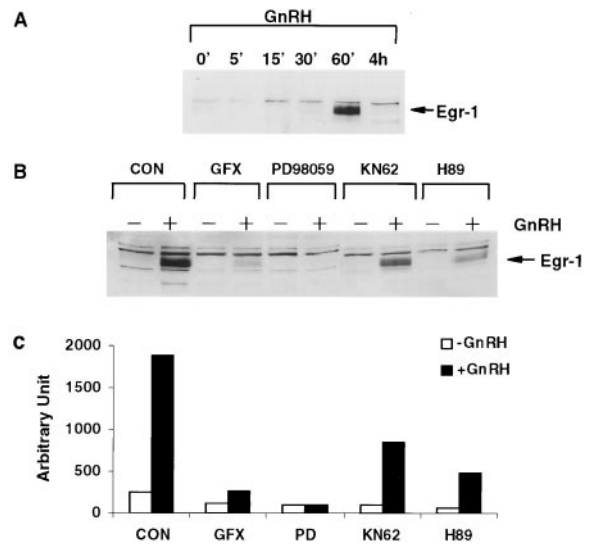


Fig. 7. Effects of Protein Kinase Inhibitors on GnRH-Induced Egr-1 Protein Expression

$\alpha T3$ cells were treated with 10 nM GnRH for the time indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an anti-Egr-1 antibody. B, $\alpha T3$ cells were preincubated with DMSO, GF109203X ($5 \mu\text{M}$), PD98059 ($50 \mu\text{M}$), KN-62 ($5 \mu\text{M}$), or H-89 ($10 \mu\text{M}$) for 1 h. Cells were then treated with or without 10 nM GnRH for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using an anti-Egr-1 antibody. C, Protein levels in panel B were quantitated using a densitometer.

contrast, PMA stimulation of Elk-1 was partially blocked by $1 \mu\text{M}$ GF109203X. PD98059 completely blocked GnRH-induced Elk-1 phosphorylation (Fig. 9B). Forskolin did not inhibit GnRH-induced Elk-1 phosphorylation.

DISCUSSION

Egr-1 is a ubiquitous transcription factor that participates in a wide range of physiological and pathophysiological processes (17). The generation of Egr-1 knockout mice revealed an unanticipated critical role for Egr-1 in the regulation of LH β gene expression (11, 12). Several studies have documented that GnRH up-regulates Egr-1 gene expression (13, 15, 16, 47). The finding of a GnRH-responsive transcription factor offers the opportunity to better understand how GnRH regulates LH biosynthesis, and it also provides a proximate target of GnRH action to help unravel signaling pathways that mediate transcriptional events.

The Egr-1 promoter contains multiple putative regulatory elements, including two Sp1 sites, five SREs with adjacent Ets-like motifs, two CREs, and Egr-1 binding sites (26, 29). The SREs in the Egr-1 promoter have been extensively studied. The distal SREs are critical for induction of Egr-1 by GH (63), platelet-derived growth factor (64), GM-CSF (65), urea (66), and stress (44). The proximal SREs are necessary for

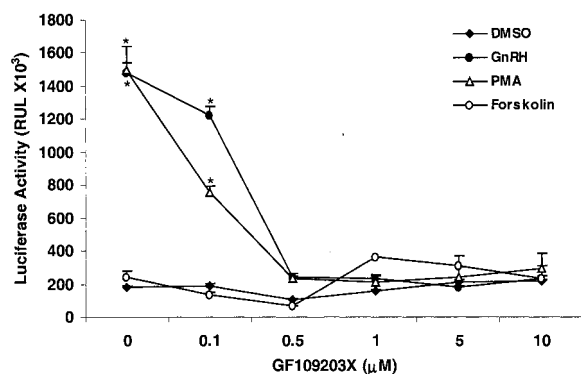


Fig. 8. Effect of Different Concentrations of GF109203X on GnRH-Induced Egr-1 Promoter Activity

α T3 cells were transiently transfected with the -442 -bp Egr-1 promoter construct ($0.5 \mu\text{g}/\text{well}$). Forty hours after the transfection, cells were preincubated with different concentrations of GF109203X as indicated for 1 h. Cells were then treated with DMSO, GnRH (10 nM), PMA (100 nM), or forskolin ($10 \mu\text{M}$) for 4–6 h. Cell extracts were prepared and assayed for luciferase activity. Values shown are the mean \pm SEM of triplicate transfection. The experiment was repeated three times, and similar data were obtained. *, $P < 0.05$ vs. DMSO-treated cells.

maximal induction by GM-CSF (32). By deletion analysis of the murine Egr-1 promoter in α T3 cells, two regions that are responsible for transcriptional activation by GnRH were localized. The first region (A: -370 to -342) contains two functional SREs. The second region (B: -116 to -73) spans a putative Ets motif with two adjacent SREs. The distal SREs are predominantly responsible for GnRH action (70–80% inducibility), but the proximal SREs and Ets binding site also confer GnRH stimulation (20–30% inducibility), suggesting a degree of redundancy or additivity in their actions. The SRF binds to the SREs in region A as evidenced by a supershift using the anti-SRF antibody. Binding of SRF to the CArG box is thought to recruit TCF family members, such as Fli-1, Elk-1, and Sap-1a, to the Ets motif sequence (35). Two complexes were observed when a proximal SRE- and Ets-containing oligonucleotide was used. The upper band is an SRF-containing complex. The lower band may represent TCF proteins. It is established that Fli-1 bound the Ets sites in the Egr-1 promoter in GM-CSF-stimulated extracts (65, 67), whereas Elk and Sap-1a were found in DNA-protein complexes of Egr-1 promoter in GH-stimulated 3T3-F442A cells (63). Although there is no direct evidence of Elk-1 binding to Ets motif, we showed that Elk-1 could be phosphorylated through the ERK pathway upon GnRH stimulation in a pituitary cell line. Activated Elk-1 has been recognized as a transcription factor that cooperatively interacts with the SRF and binds to the SRE/Ets motifs in the promoter region of various genes (40, 68, 69). Phosphorylation of Elk-1 has been reported to contribute to Egr-1 induction by urea (60), stress (61), and a calmodulin antagonist (62). In addition, GnRH has been shown to

stimulate Elk-1 activation as a downstream event of MAPK cascade (70) in human granulosa-luteal cells (71), and *c-fos* was proposed as a target gene.

It is notable that SRF bound to the SREs independent of GnRH stimulation. This finding agrees with other studies in which SRF constitutively binds SREs with no change after stimulation by growth factors or serum (65, 72–74). It is possible that the primary function of SRF in GnRH stimulation is to act as a docking element and associate with other transcription factors or to stabilize DNA binding of CREB or Ets TCF proteins, as suggested in GH induction of Egr-1 (63). Alternatively, SRF is a phosphoprotein that can be phosphorylated by p90RSK, calmodulin II and IV, and MAPK-activated protein kinase in response to growth factors, stress, or other agents (53, 75, 76). SRF phosphorylation is thought to facilitate binding of SRF to the SRE (53, 77). Therefore, SRF is also likely a direct intracellular target of signal transduction cascades.

CREB binding to the CRE is involved in the regulation of Egr-1 gene expression in response to certain stimuli (20, 41, 42). We have previously shown that GnRH-stimulated CREB phosphorylation contributes to transcriptional activation of the α -subunit gene in the pituitary (78). In support of the hypothesis that CREB may mediate GnRH regulation of Egr-1 expression, EMSA revealed that CREB constitutively binds to the CRE of the Egr-1 promoter, regardless of the presence or absence of GnRH. After GnRH stimulation, however, the binding of phospho-CREB to CRE substantially increased. Phospho-CREB binding was reduced in the presence of the PKC inhibitor, GF109203X (data not shown), suggesting that the PKC pathway is involved in GnRH-stimulated CREB phosphorylation. In addition, we show that p90RSK is also stimulated by GnRH, providing another pathway for CREB phosphorylation. Although CREB recognizes the CRE of the Egr-1 promoter, it appears that other factors also bind to this element, as a major fast-migrating band also associates with the CRE probe (Fig. 4B). The identity and function of this band remain to be determined.

An Egr-1 reporter gene containing a CRE mutation exhibited 35–40% reduction of GnRH-induced activation. Of note, this reduction was observed only with reporter constructs containing the distal SREs, implicating functional interactions between the SREs and the CRE. CREB binding protein (CBP) has been shown to interact with the C-terminal transactivation domains of the TCFs, Elk-1 and Sap-1a, and with full-length SRF (79–81). CBP is also recruited to the *c-fos* SRE through interactions between the bromodomain and Elk-1 (82). Phosphorylation of Elk-1, SRF, CREB, as well as CBP itself, may induce a conformational change that permits the transactivation domains of CBP to contact the basal transcription machinery and thus potentiate transcriptional initiation (82).

Multiple intracellular pathways, including p38/SAPK2 (20), ERK/pp90RSK (19, 24, 34, 44–46), PKC (19, 44, 45, 50), and tyrosine kinase have been shown

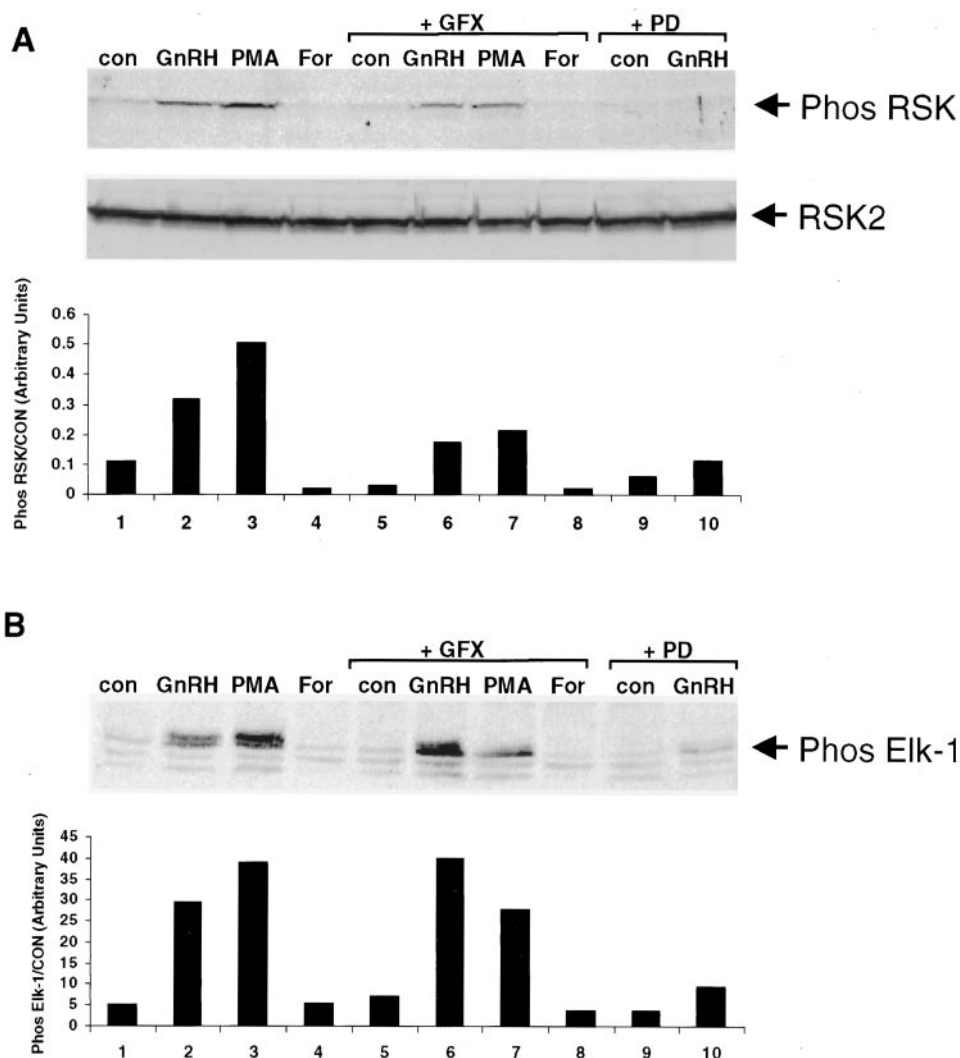


Fig. 9. GnRH Stimulates p90RSK and Elk-1 Phosphorylation in α T3 Cells

A, GnRH stimulation of p90RSK. α T3 cells were preincubated with DMSO, GF109203X (1 μ M), or PD98059 (50 μ M) for 1 h. Cells were then treated without or with GnRH (10 nM), PMA (100 nM), or forskolin (10 μ M) for 1 h. Cell extracts were prepared and subjected to Western blot analysis using an antiphospho-p90RSK antibody. An antibody against RSK2 was used as a control (*upper panel*). Protein levels were quantitated using densitometry and normalized to the control (RSK2) value (*lower panel*). B, GnRH stimulation of Elk-1. α T3 cells were preincubated with DMSO, GF109203X (1 μ M), or PD98059 (50 μ M) for 1 h. Cells were then treated without or with GnRH (10 nM), PMA (100 nM), or forskolin (10 μ M) for 1 h. Cell extracts were prepared and subjected to Western blot analysis using antiphospho-Elk-1 antibody (*upper panel*). Protein levels were quantitated using densitometry and normalized to the control (RSK2) value (*lower panel*).

to mediate Egr-1 stimulation (51). The PKC pathway appears to be necessary for GnRH stimulation of the Egr-1 gene (13, 15, 47, 48). Consistent with these studies, we found that pharmacological activation of PKC by PMA induces Egr-1 promoter activity, and a PKC inhibitor is sufficient to abolish Egr-1 stimulation by GnRH as well as PMA. Although experiments using pharmacological agents must be interpreted with caution, these findings suggest a pivotal role for the PKC pathway in Egr-1 induction and are reminiscent of similar findings with the α -subunit promoter (7, 83, 84). Transfection of PKC ζ with the Egr-1 promoter appears to increase basal and GnRH-stimulated Egr-1 pro-

motor activity (data not shown), raising the possibility that it may mediate PKC action. In addition to PKC, the ERK pathway is activated by GnRH and contributes to α -promoter stimulation (70, 85, 86). Our finding that an ERK inhibitor prevents GnRH stimulation of Egr-1 promoter activity and protein expression confirms the role of the ERK pathway in GnRH activation of Egr-1, consistent with another report that PD98059 blocks GnRH stimulation of Egr-1 mRNA (48).

Although activation of the ERK cascade by GnRH is well documented, the downstream mediators have not been well characterized. Several lines of evidence suggest that p90RSK activity is necessary for growth

factor-induced immediate-early gene expression (46, 52, 56). RSK family members are thought to influence gene expression through phosphorylation of transcription factors, such as SRF and CREB (53, 54, 87). All three members of RSK have been shown to catalyze CREB phosphorylation *in vitro* and *in vivo* (56). Similar to growth factors, GnRH was found to stimulate p90RSK activation in our study. Pretreatment with PD98059 and GF109203X suppressed GnRH-induced p90RSK phosphorylation, suggesting that PKC-Raf-MEK-ERK and p90RSK are involved in GnRH regulation of Egr-1 gene expression. Therefore, SRF and CREB may serve as direct intracellular targets of the ERK pathway in GnRH regulation of the Egr-1 gene. It will therefore be interesting to determine whether p90RSK directly phosphorylates SRF and CREB after GnRH stimulation. In addition, it is unclear whether RSK2 and RSK3 can be activated and participate in GnRH regulation of the Egr-1 gene. It should be noted that a PKC-independent ERK pathway might also contribute to GnRH stimulation of the Egr-1 gene, as Elk-1 phosphorylation was blocked by PD98059 but not by GF109203X. A recent study indicated that activation of ERK by GnRH involved two distinct signaling pathways: one is mediated by PKC and the other involves Ras activation by Src and dynamin (25, 71).

Other MAPK family members, such as p38MAPK, are activated by GnRH and contribute to GnRH induction of the *c-fos* promoter (88). Jun N-terminal kinase (JNK/SAPK1) is activated by GnRH in a PKC-independent pathway in gonadotrope cells (89, 90), and a JNK cascade appears to be necessary to elicit an LH β promoter induction (90). Future studies may yield insights into whether p38MAPK and JNK are involved in GnRH regulation of Egr-1. Although PKA and Ca⁺² kinase inhibitors appear to weakly inhibit Egr-1 promoter activity and protein expression induced by GnRH, these effects were inconsistent. Given the complex regulatory elements in the Egr-1 promoter, it is likely that more than one signaling pathway is involved in GnRH regulation of Egr-1. Our data suggest that a complicated array of signaling cascades interact with regulatory elements and transcription factors to regulate the Egr-1 promoter. In future studies, it should be informative to investigate how pulsatile GnRH is coupled to these signaling pathways and regulatory elements to dynamically control the level of Egr-1, which in turn modulates LH β gene expression.

MATERIALS AND METHODS

Plasmids

The murine Egr-1 promoter (−1,381 to +79) was amplified by PCR and subcloned into the pGL3 basic luciferase reporter construct vector (Promega Corp., Madison, WI). A series of deletion constructs (−1,072, −841, −692, −542, −482, −442, −392, −370, −342, −292, −245, −192, −142, −130, −116, and −73) was made by either restriction digestion or PCR. Reporter genes containing mutations at the SREs, Ets

binding site (−370 m), and CRE (−692 m) were constructed by overlapping PCR. The DNA sequence of the promoter region was confirmed using a dRhodamine terminator cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT) and an ABI377 automated sequencer (PE Applied Biosystems, Foster City, CA).

Cell Culture, Transfection, and Luciferase Assays

α T3 cells were grown to approximately 50–70% confluency in DMEM/F12 with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin in 12-well plates. Cells were washed with serum-free media and transfected with Egr-1 promoter-reporter constructs (0.5 μ g DNA/well) using Transfectam (Promega Corp.), according to manufacturer's instructions. Cells were incubated with the transfection mixture at 37 C for 2.5 h, and complete media were added to the cells. Forty hours after transfection, cells were treated with or without 10 nM GnRH analog (Des-Gly¹⁰, [D-Ala⁶]-GnRH ethylamide; Sigma, St. Louis, MO) for 4–6 h. Cell extracts were then harvested and analyzed for luciferase activity as described previously (78). In experiments involving protein kinase inhibitors, cells were preincubated with an inhibitor for 1 h and treated with GnRH or other reagents. All experiments were performed in triplicate and repeated at least three times. Data were presented as the mean \pm SEM. Statistical analyses were performed using the *t* test, and *P* < 0.05 was considered statistically significant.

EMSAs

Nuclear extracts were prepared from α T3 cells treated with or without GnRH for 1 h by the method of Shapiro *et al.* (91) in the presence of a protease inhibitor mixture (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and 25 mM NaF. Nuclear extracts were also obtained from human embryonic kidney TSA 201 cells grown in DMEM supplemented with 10% FBS and transfected with an empty or CREB expression vector. EMSA was performed as described previously (92). Briefly, nuclear extracts (10 μ g) were incubated with 20 fmol of ³²P-labeled oligonucleotides (Table 1) for 30 min on ice. For antibody supershift experiments, nuclear extracts were incubated with antibodies for 30 min on ice before radiolabeled probes were added. Anti-SRF, -Ets, and -CREB antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiphospho-CREB antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). DNA-protein complexes were resolved on 4% native polyacrylamide gels in 0.5 \times Tris-borate-EDTA buffer and subjected to autoradiography.

Western Blot Analyses

α T3 cells at about 80% confluency were preincubated with or without a kinase inhibitor at 37 C for 1 h and then treated with GnRH or other reagents for an additional 1 h. The same concentration of an inhibitor was maintained during GnRH stimulation. Cells were washed with ice-cold PBS and harvested. Nuclear extracts were prepared as described above. In experiments involving CREB, p90RSK, and Elk-1, cell extracts were prepared. Equal amounts of protein (nuclear or cell extracts) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose filters. The membranes were blocked with 3% nonfat milk for 1.5 h and then incubated overnight at 4 C with anti-Egr-1 (Santa Cruz Biotechnology, Inc.), phospho-CREB, phospho-p90RSK, or phospho-Elk-1 antibody (Cell Signaling Technology, Beverly, MA). Immunoreactive proteins were detected using an antirabbit horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence system (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Bands were quantitated using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Table 1. EMSA Primers

Name	Sequence			
Region A				
	-374	<i>SRE</i>	<i>SRE</i>	-334
Wild-type	5'-ACAGA <i>CCTTATTTGG</i> GCAGCG <i>CCTTATATGG</i> AGTGGCCCAA-3'			
Mutant	5'-ACAGA <i>CCTTATTAAT</i> GCAGCG <i>CCTTAAGTGG</i> AGTGGCCCAA-3'			
Region B				
	-114	<i>SRE</i>	<i>Ets</i>	<i>SRE</i>
Wild-type	5'-CCGGTCCTT <i>CCATATTAGG</i> G <i>CTTCCTGC</i> TTC <i>CCATATATGG</i> CCATGTA-3'			
Mutant	5'-CCGGTCCTT <i>CCATATGAAG</i> G <i>CTTAATGC</i> TTC <i>CCATAAGTGG</i> CCATGTA-3'			
CRE				
	-144	<i>CRE</i>		-125
Wild-type	5'-AGGGCT <i>TACAGTC</i> ACTCCGG-3'			
Mutant	5'-AGGGCT <i>TCTCATC</i> ACTCCGG-3'			

SREs, Ets binding site, and CRE are shown by *italics*. Mutations are *underlined*. Nucleotides are numbered relative to the transcription start site.

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Summer Institute on Aging Research 2002

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