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Egr-1 Is Activated by 17β-Estradiol in MCF-7 Cells by Mitogen-Activated Protein Kinase-Dependent Phosphorylation of ELK-1

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Abstract Early growth response-1 (*Egr-1*) is an immediate-early gene induced by E2 in the rodent uterus and breast cancer cells. E2 induces Egr-1 mRNA and protein levels in MCF-7 human breast cancer cells and reporter gene activity in cells transfected with pEgr-1A, a construct containing the −600 to +12 region of the Egr-1 promoter linked to the firefly luciferase gene. Deletion analysis of the Egr-1 promoter identified a minimal E2-responsive region of the promoter that contained serum response element (SRE)3 (−376 to −350) which bound Elk-1 and serum response factor (SRF) in gel mobility shift assays. Hormone-responsiveness of Egr-1 in MCF-7 cells was specifically inhibited by PD98059, a mitogen-activated protein kinase kinase inhibitor, but not by LY294002, an inhibitor of phosphatidylinositol-3-kinase (PI3-K). These results contrasted with hormone-dependent activation of the SRE in the c-fos promoter, which was inhibited by both PD98059 and LY294002. Differences in activation of the SREs in Egr-1 and c-fos were related to promoter sequence, which defines the affinities of Elk-1 and SRF to their respective binding sites. Thus, Egr-1, like c-fos, is activated through non-genomic (extranuclear) pathways of estrogen action in breast cancer cells. J. Cell. Biochem. 93: 1063–1074, 2004.

Key words: Egr-1; extranuclear; estrogens; MAPK; Elk-1

Treatment of mammalian cells with mitogens, cytokines, and differentiation inducing agents is accompanied by alterations in expression of multiple genes that play integral roles in mediating cell-specific responses [Cochran et al., 1983; Kruijer et al., 1985; Lau and Nathans, 1987; Sukhatme et al., 1987, 1988; Curran and Franza, 1988; Cohen and Curran, 1989; Sukhatme, 1990]. For example, immediate-early genes such as c-*fos* are rapidly induced (0.3–2 h) in several mammalian cell lines after treatment with mitogens. c-Fos protein is a nuclear transcription factor that forms part of the activating protein-1 (AP-1) complex and regulates AP-1-dependent gene expression [Cochran et al., 1983; Kruijer et al., 1985; Lau and Nathans, 1987; Sukhatme et al., 1987, 1988; Curran and Franza, 1988; Cohen and Curran, 1989; Sukhatme, 1990]. The early growth response-1 (*Egr-1*) gene is also a member of the immediate-early gene group of transcription factors and at least four *Egr* genes have been identified [Sukhatme, 1990]. Egr-1 is a nuclear transcription factor that contains three zinc finger motifs and, like Wilm's tumor suppressor (WT-1) and Sp family proteins, Egr-1 binds GC-rich promoter DNA sequences [Christy and Nathans, 1989; Wang and Deuel, 1992]. Egr-1 modulates transcription of multiple genes and the overall cellular responses to Egr-1 are complex and dependent on both promoter- and cell-context.

Results of several studies show that Egr-1 plays a role in apoptosis and suppresses growth of tumors derived from multiple tissues/organs [Huang et al., 1994, 1995, 1997; Muthukkumar et al., 1995; Ahmed et al., 1996; Liu et al., 1996; Nair et al., 1997]. For example, low to non-detectable levels of Egr-1 are expressed in

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several cancer cell lines including ER-negative and ER-positive breast cancer cells, whereas relatively high levels are expressed in non-transformed MCF10A and 184A1N4 mammary cells in culture [Huang et al., 1997]. Egr-1 is induced in some but not all cell lines after treatment with serum, ultraviolet light, or phorbol esters, and there were differences in Egr-1 inducibility even among ER-positive MCF-7 (inducible), ZR-75, and T47D breast cancer cell lines [Huang et al., 1997].

Egr-1, c-*fos*, and several other immediate-early genes are induced by 17β-estradiol (E2) in the rodent uterus and in MCF-7 breast cancer cells [Suva et al., 1991; Cicatiello et al., 1993; Pratt et al., 1998]. Pratt et al. reported that E2 activates Egr-1 expression in MCF-7 cells, and this is accompanied by rapid autophosphorylation of raf-1 suggesting that hormonal regulation of Egr-1 may involve rapid non-genomic (extranuclear) pathways of estrogen action which have been extensively described in multiple cell types [reviewed in Watson et al., 1998, 2002; Levin, 2002; Santen et al., 2002]. This study further investigates the molecular mechanism of E2-dependent activation of Egr-1 in MCF-7 breast cancer cells. E2 induces luciferase activity in cells transfected with pEgr-1A which contains the −600 to +12 Egr-1 promoter insert. Subsequent deletion and mutation analysis showed that a serum response element (SRE) at −376 to −350 (SRE3) was required for E2-induced transactivation, and this was accompanied by increased phosphorylation of elk-1 which interacts with serum response factor (SRF) bound to the SRE. Thus, both intermediate-early genes *Egr-1* and c-*fos* [Duan et al., 2001, 2002] are induced by E2 in MCF-7 cells through activation of elk-1 and SRF bound to specific promoter SRE motifs.

MATERIALS AND METHODS

Chemicals, Cells, and Antibodies

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely maintained in DME/F12 medium with phenol red and supplemented with 5% fetal bovine serum (FBS) plus antibiotic antimycotic solution (Sigma, St. Louis, MO) in an air–carbon dioxide (95:5) atmosphere at 37°C. For transient transfection studies, cells were grown for 1 day in DME/F12 medium without phenol red and 2.5% FBS stripped with dextran-coated charcoal. ICI 182780 was kindly provided by Dr. Alan Wakeling (AstraZenaca Pharmaceuticals, Macclesfield, UK). The kinase inhibitors PD98059, LY294002, SB202190, and SP600125 were purchased from Calbiochem (La Jolla, CA). Elk-1, actin, phospho-Elk-1, Egr-1, and SRF antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals and biochemicals were the highest quality available from commercial sources.

Cloning and Plasmids

Wild-type human ERα (hERα) expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). The SRF-luc construct contains five tandem SRF elements linked to bacterial luciferase and was purchased from Stratagene (LaJolla, CA). Dominant negative (dn) Elk-1 was provided by Dr. Roger Davis (University of Massachusetts, Worcester, MA). This construct encodes amino acid residues 1–168 of Elk-1 and lacks the activation domain. The plasmid Gal-Elk-1C was obtained from Dr. Roger Treisman (Imperial Cancer Research Center, London, UK). pEgr1-CAT plasmid, which contains the −600 to +12 5′ flanking sequence from the human *Egr-1* gene was kindly provided by Dr. Kathy Sakamoto (UCLA School of Medicine, Los Angeles, CA). pEgr-1A (−600/+12), pEgr-1B (−460/+12), pEgr-1C (−164/+12), pEgr-1D (−480/−285), pEgr-1E (−480/−324), and pEgr-1F (−480/−348) were made by PCR amplification using pEgr1-CAT as template. The PCR products were purified and ligated into pGL2 basic vector (Promega Corp., Madison, WI). Site-directed mutagenesis was performed using the two-step overlap extension PCR method. Oligonucleotides used for site-directed mutagenesis in this study are listed as follows:

pEgr-1Em1, 5′ GCA GCA CCT T**CC** TTG GAG TGG C 3′;

pEgr-1Em2, 5′ GAA CAA CCC TT**G** **C**TT GGG CAG CAC 3′;

pEgr-1Em3, 5′ GAT CCC CCG CCT **AGC** **T**AA CCC TTA TTT GG 3′;

Elk-1c (S383A), 5′ GAG CAC CCT G**GC** TCC CAAT TGC GC 3′;

Elk-1C (S389A), 5′ TGC GCC CCG T**GC** CCC GGC CAA GC 3′.

Mutations are underlined and substituted bases are indicated in bold.

Transient Transfection and Luciferase Assay

For transfection experiments, 175,000 MCF-7 cells were initially seeded in 12-well plates. Twenty-four hours after seeding, MCF-7 cells were transfected by the calcium phosphate method with Egr-1 promoter-luciferase reporter constructs, hERα expression vector, and pCDNA3/His/lacZ (Invitrogen, Carlsbad, CA) that was used as a standard reference control plasmid for determining transfection efficiencies. After 5 h, cells were shocked with 25% glycerol and washed with PBS. Fresh DME/F12 without phenol red and charcoal-stripped FBS containing DMSO or 1 nM E2 in DMSO were added to the cells and incubated for 24 h. Cells from each experiment were then harvested in 100 μl of 1× Reporter lysis buffer (Promega). Luciferase assays were performed on 30 μl of the cell extract using the Luciferase assay system (Promega). Light emission was detected on a Lumicount luminometer (Packard, Meriden, CT). β-Galactosidase assays were performed on 20 μl of cell extract using the luminescent Galacton-Plus assay kit (Tropix, Bedford, MA). The luciferase activity observed in each treatment group was normalized to β-gal activity obtained from the same sample to correct for transfection efficiencies. Data are expressed as fold induction (by E2 or other chemicals) compared to the solvent (DMSO) control.

Western Blot Assay

Cells were seeded into 60-mm tissue culture plates in DME/F12 medium without phenol red containing 2.5% charcoal-stripped FBS. After 24 h, cells were treated with 10 nM E2 and harvested at designated time points and lysed in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 10% [v/v] glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein from each treatment group were boiled in 1× Laemmli buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 0.1% bromphenol blue, 175 mM β-mercaptoethenol), separated by SDS–10% polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with Blotto (5% milk, Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and 0.05% Tween 20) and probed with primary antibodies. Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Perkin Elmer Foster City, CA).

Nuclear Extract Preparation and EMSA

MCF-7 cells were seeded in 100-mm tissue culture plates using DME/F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were treated for 1 h with DMSO or 10 nM E2. Nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Nuclear extracts obtained from different treatment groups were incubated for 20 min in HEGD buffer with poly-(dI-dC), unlabeled oligonucleotides or antibodies for supershift assays. The mixture was then incubated for additional 20 min after addition of ^{32}P-labeled oligonucleotide. Reaction mixtures were separated on 5% polyacrylamide gels (acrylamide:bis-acrylamide 30:0.8) at 140 V in 1× TBE (0.09 M Tris-HCl, 0.09 M boric acid, and 2 mM EDTA, pH 8.3). Gels were dried and protein–DNA complexes were visualized using a Storm 860 instrument (Amersham Biosciences, Piscataway, NJ). Oligonucleotides used for EMSA in this study were listed as follow (mutations are underlined and substituted bases are indicated in bold).

SRE3, 5′ AGG ATC CCC CGC CGG AAC AAC CCT TAT TTG GGC AG 3′;

mTCF, 5′ AGG ATC CCC CGC C**TA** **GCT** AAC CCT TAT TTG GGC AG 3′;

mSRF, 5′ AGG ATC CCC CGC CGG AAC AAC CCT T**GC** TTG GGC AG 3′.

RT-PCR Assay

Total RNA was extracted using Nucleospin RNA purification kit (BD Biosciences Clontech, Palo Alto, CA), following the manufacturer's instructions. An aliquot of 750 ng RNA was used as template for cDNA synthesis by incubating with oligo-d(T) primer and multiscribe reverse transcriptase (Perkin Elmer) at 48°C for 40 min. PCR amplification was performed with Taq PCR Master Mix (Promega). The following conditions were used for the PCR assays: 1 cycle of 2 min at 95°C; 34 cycles of 30 s at 95°C; 30 s at 57.5°C; 1 min at 72°C; 1 cycle of 5 min at 72°C. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Oligonucleotides used for PCR in this study include the follows: Egr-1, 5′ GAG CCG AGC GAA CAA CCC TAC GAG CAC CTG and 5′

GCG CTG AGG ATG AAG AGG TTG GAG GGT TGG; GADPH, 5′ TGT GTC CGT CGT GGA TCT GA and 5′ CCT GCT TCA CCA CCT TCT TGA; c-fos, 5′ GCT TCA ACG CAG ACT ACG AG and 5′ TAG AAG GAC CCA GAT AGG TC.

Statistical Analysis

Statistical significance was determined by ANOVA and Student's *t*-test, and the levels of probability are noted. The results are expressed as mean ± SD for at least three separate (replicate) experiments for each treatment.

RESULTS

Deletion and Mutational Analysis of the Egr-1 Gene Promoter

The results in Figure 1A show that E2 induced Egr-1 protein levels by approximately 8.2-fold, and this complements results of previous studies in MCF-7 cells which show that E2 induces Egr-1 mRNA levels [Pratt et al., 1998]. E2 did not induce luciferase activity in MCF-7 cells transfected with pEgr-1A alone; however, in cells cotransfected with ERα expression plasmid (500 ng), E2 induced luciferase activity (>7-fold), and this response was inhibited by the antiestrogen ICI 182780 (Fig. 1B). Thus, hormone-responsiveness in MCF-7 cells was observed only after cotransfection with ERα. Similar results have previously been reported for multiple E2-responsive plasmids activated through nuclear or extranuclear pathways of estrogen action, and this is related to limiting levels of ERα in transfected cells that overexpress the plasmids [Dubik and Shiu, 1988; Berry et al., 1989; Savouret et al., 1991; Cavailles et al., 1993; Zacharewski et al., 1994; Krishnan et al., 1995; Webb et al., 1995; Porter et al., 1997; Sathya et al., 1997; Duan et al., 2001, 2002] However, it was also observed that higher concentrations of E2 also significantly induced luciferase activity in MCF-7 cells transfected only with pEgr-1A (no hERα cotransfection) (Fig. 1C). This has also been reported for hormone-dependent activation of constructs containing c-fos promoter inserts which are activated through kinase-dependent pathways in MCF-7 cells [Duan et al., 2001, 2002].

The −600 to +12 region of the Egr-1 promoter contains several potential E2-responsive motifs including a distal GC-rich motif that could be activated by ERα/Sp1 (nuclear), multiple SREs (SRE1-4), and cAMP response element (CRE) that can be hormonally activated through non-genomic (extranuclear) pathways [Aronica and Katzenellenbogen, 1991, 1993; Aronica et al., 1994; Watson et al., 1998, 2002; Dong et al., 1999; Castro-Rivera et al.,

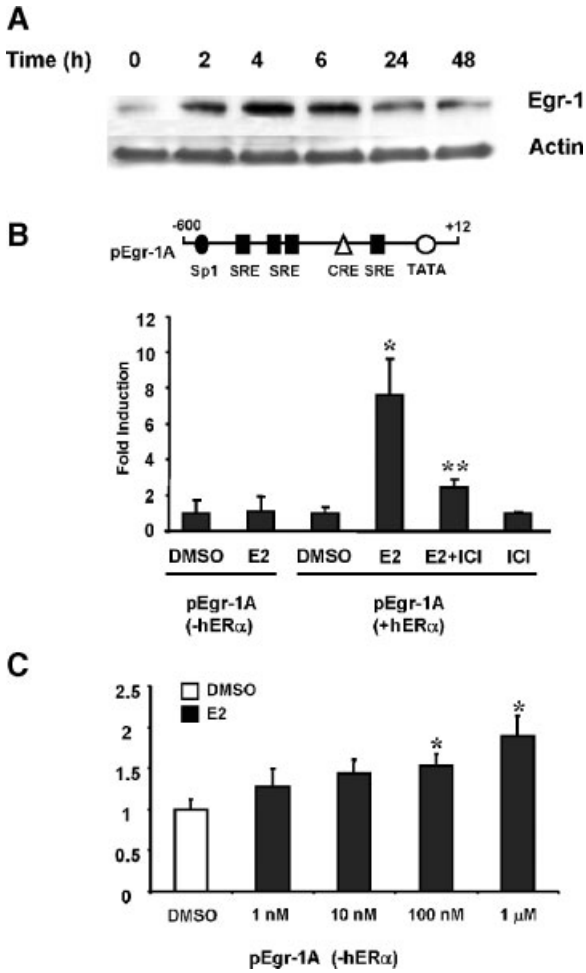

Fig. 1. Hormone-responsiveness of early growth response-1 (Egr-1) in MCF-7 cells. **A**: Induction of Egr-1 protein by E2. MCF-7 cells were treated with 10 nM E2 for different times and levels of Egr-1 protein were determined by Western blot analysis as described in the ''Materials and Methods.'' Actin protein was used as a loading control, and these cells were not cotransfected with ERα. **B**: Hormone activation of pEgr-1A. MCF-7 cells were transfected with pEgr-1A with or without human ERα (hERα) expression plasmid, treated with DMSO, E2, ICI 182780, or their combination, and luciferase activity was determined as described in the ''Materials and Methods.'' **C**: Activation of Egr-1A in the absence of cotransfected hERα. Cells were transfected with Egr-1A, treated with 1–1,000 nM E2, and luciferase activity was determined as described in the ''Materials and Methods.'' Results are expressed as mean ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction by E2 (*) or inhibition by ICI 182780 (**) is indicated.

2001; Duan et al., 2001; Levin, 2002; Santen et al., 2002]. Transfection studies in MCF-7 cells with pEgr-1A (−600 to +12), pEgr-1B (−460 to +12), and pEgr-1C (−164 to +12) (Fig. 2A) show that the upstream GC-rich and downstream CRE and SRE1 motifs are not necessary for hormone-inducibility suggesting the SREs 2–4 are necessary for this response. The E2-responsiveness of several 3′-deletion constructs containing SREs 2–4 (pEgr-1D, −480 to −285), SRE4 and 3 (pEgr-1E, −480 to −324), and SRE4 (pEgr-1F, −480 to −376) were also investigated in MCF-7 cells (Fig. 2B) and induction by E2 was observed only for the former two constructs. These results suggest that SRE3 was required for E2-induced transactivation. Mutation analysis of SRE3 was determined using constructs containing selective mutations in the SRF (SRE2 and SRE3) and TCF (SRE3) motifs. E2-induced transactivation was observed in cells transfected with pEgr-1Em1 which contained a mutation in an adjacent SRF binding site. However, induction was not observed in cells transfected with constructs containing SRE3 mutations in the SRF or TCF sites (pEgr-1Em2 and pEgr-1Em3) (Fig. 2C). These results indicate that SRE3 is the major E2-responsive motif in the Egr-1 gene promoter and that TCF and SRF motifs are required.

Protein Interactions With SRE3

Nuclear extracts from DMSO and E2-treated MCF-7 cells were incubated with [^{32}P]SRE3 and analyzed in a gel mobility shift assay (Fig. 3). In this gel, two major specifically-bound retarded bands were formed (see arrow) using DMSO and E2-treated extracts (lanes 2 and 6). Coincubation of both extracts with [^{32}P]SRE3 and Elk-1 (lanes 3 and 7) or phospho-Elk-1 (lanes 4 and 8) antibodies gave supershifted bands (SS→) indicating that both forms of Elk-1 were associated with the SRE oligonucleotide. Although the overall intensities of the retarded bands were comparable using both E2- and solvent (DMSO)-treated nuclear extracts, the SS phospho–Elk-1 complex was more intense using the hormone-treated extracts (lane 4 vs. lane 8). In competition experiments with unlabeled oligonucleotides (lanes 9–11), mutant oligonucleotides in the TCF (m1-SRE3) and SRF (m2-SRE3) sites only slightly decreased the more and less mobile retarded bands, respectively. In contrast, competition with the wild-type SRE3 oligonucleotide resulted in complete loss of both retarded bands. Antibody supershift experiments were also carried out using [^{32}P]SRE3 and nuclear extracts from DMSO- and E2-treated cells with SRF antibody. SRF antibody can also induce formation of a SS complex in both treatment groups (data not shown). These data are consistent with E2-induced phosphorylation of Elk-1 and interaction of SRF and phospho-Elk1 on SRE3 in the Egr-1 gene promoter, and this was comparable with SRF/Elk-1 interactions with the SRE in the c-fos gene promoter [Duan et al., 2001].

Role of Elk-1 in Activation of *Egr-1* Gene Expression

The role of Elk-1 in activation of Egr-1 was further investigated in the MCF-7 cells transfected with pEfg-1D and increasing amounts of dn expression plasmid for Elk-1 (50–500 ng) (Fig. 4A). dn-Elk-1 inhibits E2-induced activation of pEgr-1D and confirms the role of Elk-1 in activation of SRE3. GAL4-Elk-C contains the C-terminal region of Elk-1 (amino acids 307–428) fused to the DNA binding domain of the yeast GAL4 protein. The Elk-C region can serve as a transactivation domain [Marais et al., 1993; Treisman, 1996], and in the presence of cotransfected ERα, E2 induces reporter gene activity in MCF-7 cells transfected with GAL4-Elk-C and a construct containing five copies of the GAL4 response element linked to a bacterial luciferase reporter gene ($pGAL4_5$) (Fig. 4B). This construct is also induced by E2 alone (*ca.* 2-fold), but is enhanced by cotransfection with ERα due to overexpression of the reporter construct and limiting levels of endogenous ERα [Duan et al., 2001]. This hormone-induced response was inhibited by the MAPK kinase inhibitor PD98059 but not by 20 μM SB202190 or 25 μM SP600125 which inhibit p38 and jun N-terminal kinase, respectively. These results are consistent with hormonal activation of the ras-MAPK pathway in breast cancer cells [Migliaccio et al., 1996, 1998]. The results illustrated in Figure 4C compared the differences in hormone-induced activation of wild-type Elk-C and constructs containing S383A and S389A mutations. E2 activates wild-type GAL4-ElkC and partially activates the S389A mutant but not the S383A mutant, and this pattern of activation was similar to that observed for serum activation of Elk-1 in NIH 3T3 cells [Marais et al., 1993]. The results in Figure 4D demonstrate that E2 induces phosphorylation of Elk-1, and this is

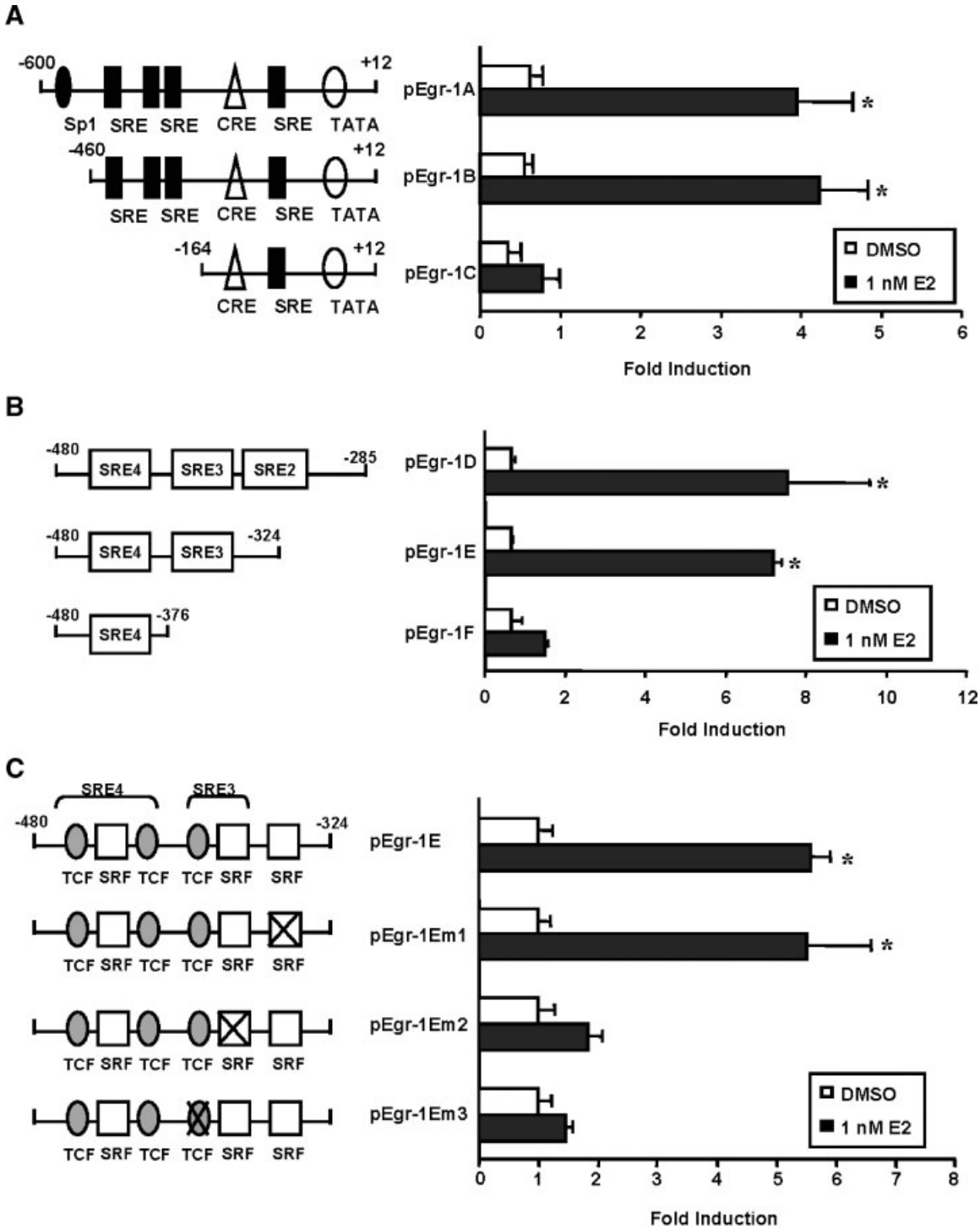

Fig. 2. Deletion and mutation analysis of the Egr-1 gene promoter. Deletion (**A**, **B**) and mutation (**C**) analysis of pEgr-1A. MCF-7 cells were transfected with pEgr-1 constructs and hERα expression plasmid, treated with DMSO or 1 nM E2, and luciferase activity was determined as described in the ''Materials and Methods.'' Results are expressed as mean ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction by E2 is indicated by an asterisk.

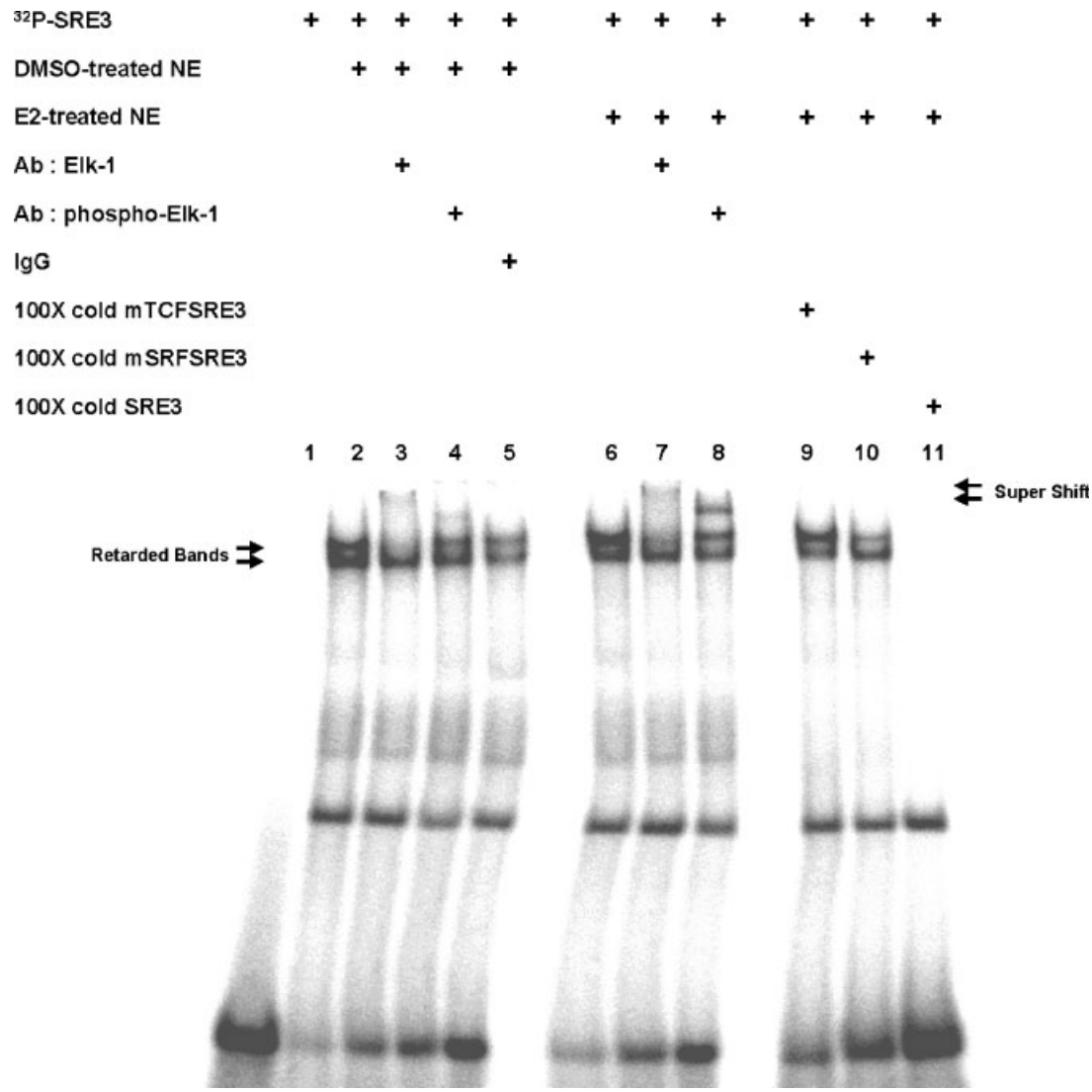

Fig. 3. Gel mobility shift assay of serum response element (SRE)3–protein interactions. **A**: Interactions of nuclear extracts with [^{32}P]SRE3. [^{32}P]SRE3 was incubated with nuclear extracts from MCF-7 cells treated with DMSO or E2 and coincubated with Elk-1, phospho-Elk-I antibodies, or non-specific IgG or unlabeled oligonucleotides (100-fold excess), and analyzed by gel mobility shift assay as described in the ''Materials and Methods.'' Retarded and supershifted (SS) bands are indicated with arrows.

inhibited by PD98059 but not SB02190, confirming the role of MAPK in this response.

However, previous studies indicate that the SRE in the c-fos gene promoter is also activated through phosphatidylinositol-3-kinase (PI3-K) which is upregulated by E2 in MCF-7 cells [Duan et al., 2001]. Results in Figure 5A show that the MAPK inhibitor PD98059 inhibits E2-induced transactivation in MCF-7 cells transfected with pEgr-1E, whereas this response is not blocked by LY294002, an inhibitor or PI3-K. As a positive control, LY294002 but not PD98059 inhibited E2-dependent activation of a construct containing five SRF elements (SRF-luc) in MCF-7 cells (Fig. 5B) as previously reported [Duan et al., 2002]. Induction of Egr-1 mRNA levels by E2 (Fig. 5C) were also inhibited by PD98059 and not LY294002 confirming that hormonal activation of Egr-1 is dependent on ERα and kinase-dependent activation of MAPK. In contrast, hormone-dependent activation of c-fos is due to activation of both MAPK and PI3-K pathways [Duan et al., 2001, 2002], and inhibitors of these pathways decrease induction of c-fos mRNA in MCF-7 cells (Fig. 5C).

DISCUSSION

Egr-1 is an immediate-early gene induced by mitogens in mammalian cells, and Egr-1 acts as a transcription factor that modulates expres-

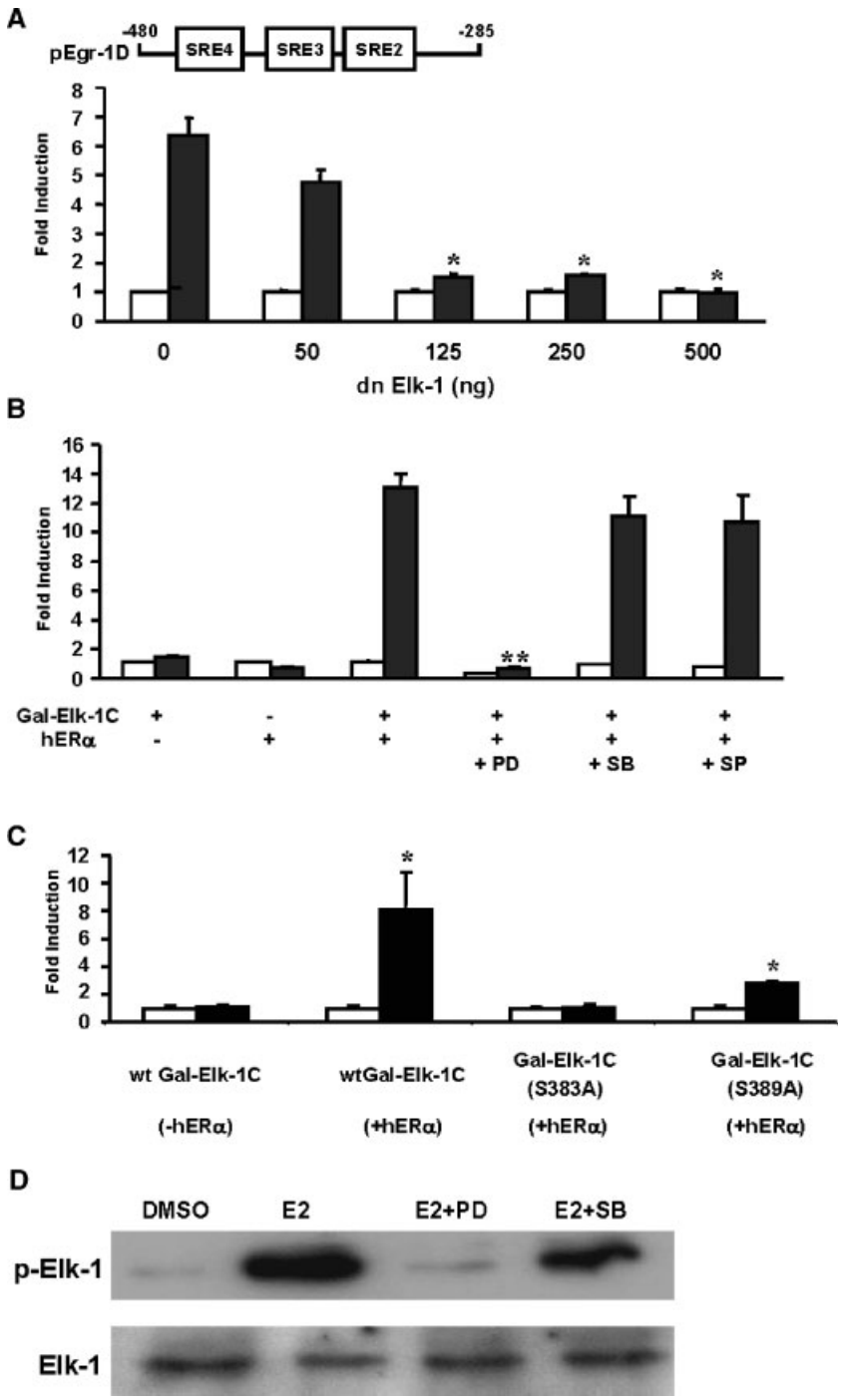

Fig. 4. Role of Elk-1 phosphorylation in activation of Egr-1. **A**: Effects of dominant negative (dn) Elk-1. MCF-7 cells were transfected with pEgr-1D, treated with DMSO or 1 nM E2, co-transfected with different amounts of dnElk-1 expression plasmid, and luciferase activity was determined as described in the ''Materials and Methods.'' Significant ($P < 0.05$) inhibition of E2-induced luciferase activity by dnElk-1 is indicated by an asterisk. **B**: Hormone activation of GAL4-Elk-1C/pGAL4$_5$. MCF-7 cells were transfected with GAL4-Elk-1C/pGAL4$_5$, hERα expression plasmid, DMSO, 10 nM E2 and kinase inhibitors (50 μM PD98059, 20 μM SB202190, and 25 μM SP600125), and luciferase activities were determined as described in the ''Materials and Methods.'' Significant ($P < 0.05$) inhibition of E2-induced activity is indicated (**). **C**: Activation of GAL4-Elk-1C. MCF-7 cells were transfected with pGAL4$_5$, wild-type and mutant GAL4-Elk-1C, (±) hERα expression plasmid, treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the ''Materials and Methods.'' Significant ($P < 0.05$) induction by E2 is indicated by an asterisk. **D**: Phosphorylation of Elk-1. MCF-7 cells were treated with DMSO, 10 nM E2 alone, or in combination with 50 μM PD98059 or 20 μM SB202190, and whole cell lysates were examined by Western blot analysis for Elk-1 and phospho-Elk-1 proteins. Similar results were observed in duplicate experiments. Results in A, B, and C are mean ± SD for three replicate experiments for each treatment group.

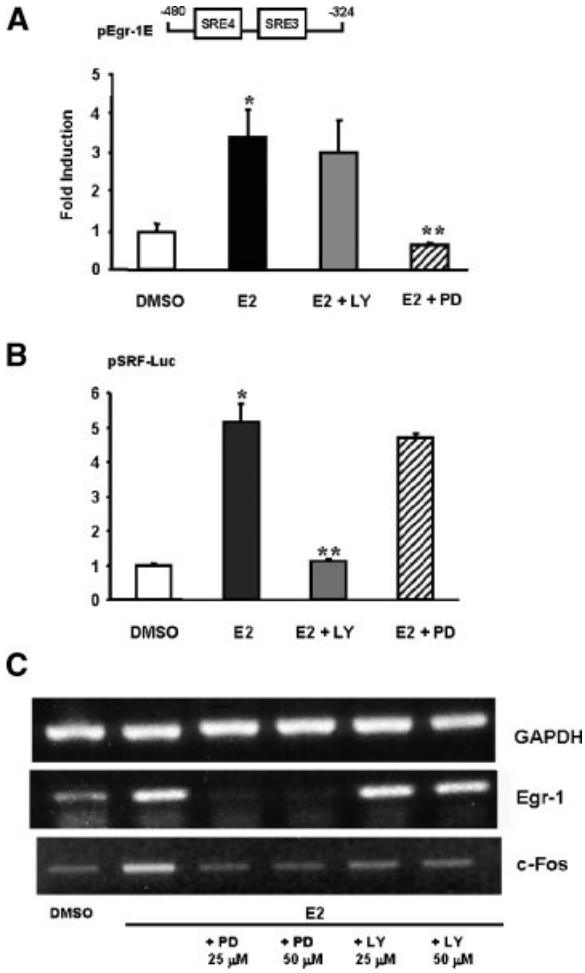

Fig. 5. Effects of PD98059 and LY294002 on hormone-induced activation of Egr-1 and comparative SRE sequences. **A**: Activation of pEgr-1E. MCF-7 cells were transfected with pEgr-1E, treated with DMSO, 10 nM E2 alone, or in combination with 25 μM PD98059 or 50 μM LY294002, and luciferase activity was determined as described in the ''Materials and Methods.'' Results are mean ± SD for three replicate experiments for each treatment group, and significant ($P < 0.05$) inhibition in cotreatment groups is indicated (*). **B**: Induction of serum response factor (SRF)-luc by E2. MCF-7 cells were transfected with SRF-luc, treated with E2, DMSO or kinase inhibitor as indicated in Figure 5A (above), and luciferase activity determined as described in the ''Materials and Methods.'' Results are mean ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction (*) or inhibition (**) is indicated. **C**: Induction of pEgr-1 and c-fos mRNA. MCF-7 cells were treated with DMSO, 10 nM E2 alone, or in combination with 25–50 μM PD98059 or 25–50 μM LY294002, and induction responses were determined by RT-PCR as described in the ''Materials and Methods.'' Similar results were observed in duplicate experiments.

sion of several genes [Cochran et al., 1983; Kruijer et al., 1985; Lau and Nathans, 1987; Sukhatme et al., 1987, 1988; Curran and Franza, 1988; Cohen and Curran, 1989; Sukhatme, 1990]. Several studies also suggest that *Egr-1* can act as a tumor suppressor gene in some cells. For example, in a subclone of human HT1080 fibrosarcoma cells, overexpression of Egr-1 inhibited transformed growth and [^{3}H]thymidine uptake and suppressed the rate of tumor growth in athymic nude mice bearing HT1080 xenografts [Huang et al., 1995]. Expression of Egr-1 was also relatively high in non-tumorigenic MCF-10A and 184A1N4 immortalized mammary epithelial cells, but low to non-detectable in ER-negative and ER-positive (ZR-75, T47D, and MCF-7) breast cancer cells lines. A similar pattern of Egr-1 expression was also observed in rat mammary tissue (high) and mammary tumors (low), suggesting that loss of Egr-1 expression may be required for development of breast cancer.

Pratt et al. previously reported that E2 induced *Egr-1* gene expression in MCF-7 cells this was accompanied by rapid autophosphorylation of raf-1. In this study, E2 also induced *Egr-1* gene expression (mRNA and protein) in MCF-7 cells (Figs. 1A and 5B), and the mechanism of this response was further investigated using a series of constructs containing Egr-1 promoter inserts. The −600 to +12 region of the Egr-1 gene promoter contains a GC-rich site, multiple SREs, and a CRE. Previous studies indicate that both SRE and CRE motifs are hormone-responsive through ERα-dependent extranuclear induction of the src-ras-MAPK and PKA pathways [Aronica and Katzenellenbogen, 1991, 1993; Aronica et al., 1994; Migliaccio et al., 1996, 1998; Dong et al., 1999; Castro-Rivera et al., 2001; Duan et al., 2001]. In contrast, the more distal GC-rich Sp1 binding site could be activated by the non-classical nuclear ERα/Sp1 pathway [Porter et al., 1997]. Deletion analysis (5′- and 3′-) of the Egr-1 gene promoter (Fig. 2A,B) indicates that SRE3 and SRE4 are E2-responsive and further mutation analysis (Fig. 2C) demonstrates that E2-responsiveness is linked to the TCF and SRF motifs within SRE3.

Previous studies in this laboratory showed that E2 also induced c-*fos* gene expression in MCF-7 cells through activation of a proximal SRE through the ras-MAPK pathway [Duan et al., 2001]. Results in Figures 3 and 4 confirm that hormone-dependent activation of SRE3 in the Egr-1 promoter is also accompanied by Elk-1 phosphorylation and is inhibited by dn Elk-1 expression. Thus, both immediate-early genes *Egr-1* and c-*fos* are induced by E2 through

activation of ras-MAPK by extranuclear pathways in breast cancer cells [Duan et al., 2001].

Recent studies have shown that Egr-1 is regulated, in part, through the MAPK pathway in several cell lines [Dziema et al., 2003; Jones and Agani, 2003; Russell et al., 2003]. For example, in rat granulosa cells, gonadotropin-dependent upregulation of Egr-1 is dependent on multiple factors including MAPK and protein kinase A-dependent phosphorylation of factors associated with SRE1 and other proximal motifs [Russell et al., 2003]. Activation of the MAPK pathway is also required for light-induced upregulation of Egr-1 in the suprachiasmatic nucleus of mice [Dziema et al., 2003] and hyperoxia-induced expression of Egr-1 in mouse alveolar carcinoma cells [Jones and Agani, 2003]. In the latter cell line, the MAPK inhibitor PD98059 blocked hyperoxia-induced expression of Egr-1, whereas PI3-K and p38 MAPK inhibitors had no effect. In the rat anterior pituitary gland and primary neonatal rat cardiomyocytes, hormonal activation of Egr-1 was MAPK-dependent and in cardiomyocytes, SREs were identified as putative hormone-responsive motifs [De Jager et al., 2001; Man and Carter, 2003]. In breast cancer cells, activation of PI3-K by E2 has been identified as an important pathway for proliferation of MCF-7 cells [Castoria et al., 2001; Sun et al., 2001; Duan et al., 2002]. Moreover, E2-mediated induction of c-fos through the SRE involved simultaneous activation of src-MAPK and src-PI3-K pathways where PI3-K activates the SRF [Castoria et al., 2001; Duan et al., 2001]. We therefore investigated the role of PI3-K in the activation of Egr-1 mRNA expression by E2 (Fig. 5C) and luciferase activity in cells transfected with pEgr-1E (Fig. 5A). The results show that for both responses, E2-induced transactivation was inhibited by PD98059 but not by the PI3-K inhibitor LY294002. Since E2 activates both MAPK and PI3-K pathways in breast cancer cells [Migliaccio et al., 1996, 1998; Castoria et al., 2001; Duan et al., 2001; Dziema et al., 2003; Jones and Agani, 2003], the differential effects of the latter pathway on activation of Egr-1 and fos through their respective SREs may be due, in part, to promoter context.

Ling et al. [1998] investigated interactions of wild-type and variant Elk-1 and SRF with different SREs to form transcriptionally-active ternary complexes. One type of SRE which is characteristic of the motif in the c-fos promoter contains a "strong" SRF site (CArG) and a weak TCF (ets) site (Fig. 6). In this model, SRF binds the SRE and recruits Elk-1, and both MAPK and PI3-K inhibitors block SRE-dependent transactivation. Another type of combined ets and CArG (CECI) motif has a high affinity TCF (or ets) site next to a weak CArG (SRF) site [Ling et al., 1998] (Fig. 6). In this model, Elk-1 binds the SRE and subsequently recruits SRF to form the transcriptionally-active ternary complex. The TCF site in the Egr-1 promoter is identical to the corresponding "strong" motif in the CECI promoter. Thus, hormone-induced transactivation of Egr-1 requires MAPK-dependent activation of Elk-1 which interacts with a "strong" TCF site, and subsequent recruitment of SRF is not dependent on activation through the PI3-K pathway. Differential activation of c-fos and Egr-1 is also consistent with the growth-promoting activities of both c-fos and the PI3-K pathway in breast cancer cells [Castoria et al., 2001; Sun et al., 2001; Duan et al., 2002], whereas Egr-1 is associated with suppression of breast cancer cell growth [Huang et al., 1995]. In contrast, there is evidence that Egr-1 may enhance formation and growth of prostate cancer [Baron et al., 2003], and current studies are investigating the mechanisms that distinguish between the differential effects of

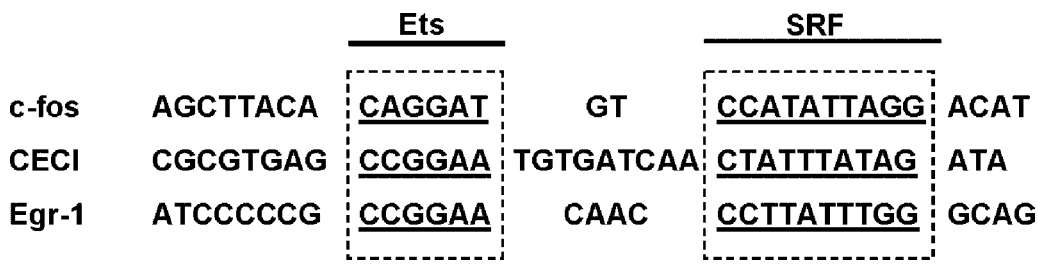

Fig. 6. Comparative SREs. The SREs in the c-fos, CECI, and Egr-1 gene promoters are given and the Ets (Elk-1 site) and SRF motifs are indicated [Ling et al., 1998].

Egr-1 in hormone-dependent breast and prostate cancer.

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